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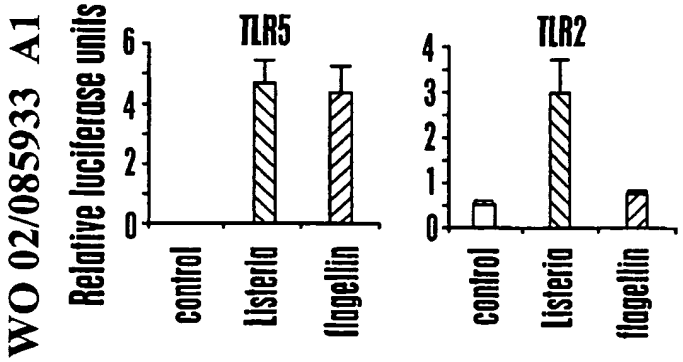
(54) Title: TOLL-LIKE RECEPTOR 5 LIGANDS AND METHODS OF USE



(57) Abstract: The invention provides an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence GAVQNRFNSAIT, or a modification thereof, and having toll-like receptor 5 (TLR5) binding. Methods of inducing an immune response are also provided.

TOLL-LIKE RECEPTOR 5 LIGANDS AND METHODS OF USE

BACKGROUND OF THE INVENTION

Cancer is the second leading cause of death in the United States, accounting for one in every four deaths. This year, it is expected that over 1500 Americans will die of cancer each day and that a million new cases of cancer will be diagnosed. The most common treatments for cancer are surgery, radiation and chemotherapy. According to the American Cancer Society, immunotherapy can be considered as the "fourth modality" in the treatment of cancer. Immunotherapy is treatment that stimulates one's own immune system to fight cancer.

Cancer is a group of diseases characterized by uncontrolled growth of abnormal cells of the body. All types of cancer involve the malfunction of genes that control cell growth and division. Some of these genes become incorrectly regulated, resulting in over- or under-production of a particular protein, while others become mutated, resulting in unusual or abnormal proteins that alter normal cellular functions. These abnormal proteins, referred to as "tumor cell antigens," should be recognized and destroyed by an individual's immune system as "foreign" antigens.

However, the immune system of a cancer patient may ignore these tumor antigens and be unresponsive to the growing tumor. Using immunotherapy approaches, such as cancer vaccines and immune system modulators, an individual's immune system can be induced to mount a potent immune response against tumor cell antigens,

resulting in elimination of cancer cells. A cancer vaccine can contain a tumor cell antigen that stimulates the immune system to recognize and destroy cells which display that antigen. Treating an individual with such a cancer vaccine can result in a humoral response, which involves producing antibodies that recognize and target tumor cells for destruction and a cellular response, which involves producing cytotoxic T cells that recognize and destroy tumor cells directly, or both responses. It can be desirable to obtain both a humoral and cellular immunity response during immunotherapy because both arms of immune response have been positively correlated with beneficial clinical responses. To help stimulate either or both humoral and cellular immune responses, a cancer vaccine can be combined with an adjuvant, which is a substance that stimulates a general immune response.

The potency of cancer vaccines is greatly enhanced by the use of adjuvants. The selection of an adjuvant for use with a particular vaccine can have a beneficial effect on the clinical outcome of vaccination. Some vaccines are ineffective in the absence of an adjuvant. Effectiveness of a vaccine may be particularly troublesome when the vaccine is produced from self antigens such as those required for cancer vaccines or other non-infectious disease vaccines. In view of the beneficial effects of adjuvants in vaccine formulations, it is surprising that only one type of adjuvant, aluminum-salt based adjuvants, are currently in wide use in United States-licensed vaccines.

Thus, there exists a need for more and improved immunological adjuvants. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence GAVQNRFNSAIT, or a modification thereof, and having toll-like receptor 5 (TLR5) binding. Methods of inducing an immune response are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows NF-κB activation and TNFα production in cells expressing CD4-TLR4 or CD4-TLR5.

Figure 2 shows selective induction of TLR5-stimulated activation of NF-κB by *P. aeruginosa* and *L. monocytogenes* cultures compared to LPS and lipopeptide.

Figure 3 shows the purification of a TRL5-stimulating activity from *L. monocytogenes* culture supernatant.

Figure 4 shows the identification by mass spectrometry of flagellin as a TLR5-stimulating activity.

Figure 5 shows that flagellin expression in bacteria reconstitutes TLR5-stimulating activity.

Figure 6 shows systemic induction of IL-6 in wild type mice treated with purified flagellin.

Figure 7 shows a comparison of flagellin amino acid sequences from 22 species of bacteria and a consensus sequence of amino acid residues conserved across species.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to flagellin derived peptides that exhibit immunomodulatory activity and to methods of inducing an immune response through activation of toll-like receptor 5 (TLR5). The identification of active flagellin peptides and their corresponding receptor, TLR5, expands the available treatment methods for inducing an immune response. Moreover, the identification of active flagellin peptides and their cognate receptor allows the identification of immunomodulatory compounds.

In one embodiment, the invention is directed to immunomodulatory flagellin peptides that bind to TLR5 and induce a TLR5-mediated activity. The peptides can be used, for example, to effectively stimulate an immune response or ameliorate a pathological condition by administration of immunomodulatory flagellin peptides and combinations of such peptides with antigens and other immunomodulatory molecules. Full length flagellin polypeptides are also used in the methods of the invention to stimulate an immune response. An advantage of the immunomodulatory flagellin peptides of the invention is that they provide the specificity of

flagellin together with the availability of rapid and efficient methods for recombinant and chemical synthesis of peptides. The immunomodulatory flagellin peptides of the invention can therefore be combined with numerous well known modes of administration for the treatment of a wide variety of pathological conditions.

In another embodiment, the invention provides a method of inducing an immune response in an individual by administering a vaccine containing an immunomodulatory flagellin peptide of the invention and an antigen. An immunomodulatory flagellin peptide of the invention functions to stimulate an innate immune response. The innate immune response involves the production of immunomodulatory molecules that beneficially promote the adaptive immune response. The adaptive immune response includes both humoral and cell-mediated immune responses to antigen. Thus, a flagellin peptide functions to boost either or both humoral and cell-mediated immune responses against the antigen. A boost in an immune response causes a general increase in immune system activity that can result in the destruction of foreign or pathologically aberrant cells that otherwise could have escaped the immune response.

As used herein, the term "flagellin" is intended to mean a flagellin polypeptide contained in a variety of Gram-positive or Gram-negative bacterial species. The nucleotide and amino acid sequences of flagellin from 22 bacterial species are depicted in Figure 7. The nucleotide sequences encoding the listed flagellin polypeptides are publically available in the NCBI Genbank database. The flagellin sequences from

these and other species are intended to be encompassed by the term flagellin as used herein. Therefore, the sequence differences between species is included within the meaning of the term.

As used herein, the term "peptide" is intended to mean two or more amino acids covalently bonded together. The term "flagellin peptide" is intended to mean a peptide or fragment encoded by a portion of the nucleotide sequence or having a portion of the amino acid sequence which exhibits substantially the same sequence identity to the flagellin sequences as described above and identified in Figure 7 and binds to toll-like receptor 5 (TLR5). For example, a flagellin peptide amino acid sequence is about 65% or greater in sequence identity to a portion of the *S. Typhimurium*1 sequence, GAVQNRFNSAIT, identified as SEQ ID NO:2, encoded by the nucleic acid sequence identified as SEQ ID NO:1. Therefore, flagellin peptides having amino acid substitutions that do not substantially alter TLR5 binding are included within the definition of a flagellin peptide. For example, flagellin peptides which contain one or more alanine substitutions and have substantially the same TLR5 binding activity as the flagellin peptide identified as SEQ ID NO:2 are included within the definition of a flagellin peptide. Exemplary flagellin peptides containing alanine substitutions and having substantially the same TLR5 binding activity as the flagellin peptide identified as SEQ ID NO:2 include, for example, GAVANRFNSAIT and GAVQNAFNSAIT. Flagellin peptides consisting of greater than twelve amino acids and having TLR5 binding activity can similarly contain amino acid substitutions, so long as such substituted

peptides retain substantially the same TLR5 binding activity. Examples of such flagellin peptides containing substitutions of various amino acid residues with alanine include ADTRDLGAVQNRFNSAIT, VDARDLGAVQNRFNSAIT and VDTADLGAVQNRFNSAIT. A flagellin peptide of the invention does not include a full length flagellin polypeptide. A flagellin peptide is intended to include molecules which contain, in whole or in part, non-amide linkages between amino acids, amino acid analogs and mimetics. Similarly, a flagellin peptide also includes cyclic peptides and other conformationally constrained structures. A flagellin peptide of the invention includes polypeptides having several hundred or more amino acid residues and can contain a heterologous amino acid sequence.

The term flagellin peptide specifically excludes fragments of flagellin described in Newton et al. Science, 244:70-72 (1989); Kuwajima, G., J. Bacteriol. 170:3305-3309 (1988); McSorley et al., J. Immunol. 164:986-993(2000); and Samatey et al. J. Struct. Biol. 132:106-111 (2000).

As used herein, term "immunomodulatory flagellin peptide," is intended to mean a peptide or fragment having a portion of the amino acid sequence which exhibits substantially the same sequence identity to the flagellin sequences as described above and shown in Figure 7 and binds to toll-like receptor 5 (TLR5). For example, an immunomodulatory flagellin peptide amino acid sequence is about 65% or greater in sequence identity to a portion of the *S. Typhimurium*1 sequence, GAVQNRFNSAIT, identified as SEQ ID NO:2, encoded by the nucleic acid sequence identified as SEQ ID NO:1.

Therefore, immunomodulatory flagellin peptides having amino acid substitutions that do not substantially alter TLR5 binding are included within the definition of an immunomodulatory flagellin peptide. For example, immunomodulatory flagellin peptides which contain one or more alanine substitutions and have substantially the same TLR5 binding activity as the flagellin peptide identified as SEQ ID NO:2 are included within the definition of a flagellin peptide. Exemplary immunomodulatory flagellin peptides containing alanine substitutions and having substantially the same TLR5 binding activity as the flagellin peptide identified as SEQ ID NO:2 include, for example, GAVANRFNSAIT and GAVQNAFNSAIT. Immunomodulatory flagellin peptides consisting of greater than twelve amino acids and having TLR5 binding activity can similarly contain amino acid substitutions, so long as such substituted peptides retain substantially the same TLR5 binding activity. Examples of such immunomodulatory flagellin peptides containing substitutions of various amino acid residues with alanine include ADTRDLGAVQNRFNSAIT, VDARDLGAVQNRFNSAIT and VDTADLGAVQNRFNSAIT. An immunomodulatory flagellin peptide of the invention does not include a full length flagellin polypeptide. An immunomodulatory flagellin peptide is intended to include molecules which contain, in whole or in part, non-amide linkages between amino acids, amino acid analogs and mimetics. Similarly, an immunomodulatory flagellin peptide also includes cyclic peptides and other conformationally constrained structures. An immunomodulatory flagellin peptide of the invention includes polypeptides having several hundred or more

amino acid residues and can contain a heterologous amino acid sequence.

An immunomodulatory flagellin peptide, polypeptide or modification thereof, of the invention binds to toll-like receptor 5 (TLR5) and induces a TLR5-mediated response. It is understood that minor modifications can be made without destroying the TLR5 binding activity, TLR5-mediated response stimulating activity or immune response modulating activity of an flagellin peptide or polypeptide and that only a portion of the primary structure may be required in order to effect activity. Such modifications are included within the meaning of the terms flagellin polypeptide and flagellin peptide so long as TLR5 binding activity, TLR5 response stimulating or immune response stimulating activities are retained. Further, various molecules can be attached to flagellin polypeptides and peptides, including for example, other polypeptides, carbohydrates, nucleic acids or lipids. Such modifications are included within the definition of the term.

Minor modifications of flagellin polypeptide and peptides having at least about the same TLR5 binding activity, TLR5 response stimulating or immune response stimulating activity as the referenced polypeptide or peptide include, for example, conservative substitutions of naturally occurring amino acids and as well as structural alterations which incorporate non-naturally occurring amino acids, amino acid analogs and functional mimetics. For example, a Lysine (Lys) is considered to be a conservative substitution for the amino acid Arg. Similarly, a flagellin peptide containing mimetic

structures having similar charge and spacial arrangements as reference amino acid residues would be considered a modification of the reference polypeptide or peptide so long as the peptide mimetic exhibits at least about the same activity as the reference peptide.

As used herein, the term "amino acid" is intended to mean both naturally occurring and non-naturally occurring amino acids as well as amino acid analogs and mimetics. Naturally occurring amino acids include the 20 (L)-amino acids utilized during protein biosynthesis as well as others such as 4-hydroxyproline, hydroxylysine, desmosine, isodesmosine, homocysteine, citrulline and ornithine, for example. Non-naturally occurring amino acids include, for example, (D)-amino acids, norleucine, norvaline, p-fluorophenylalanine, ethionine and the like. Amino acid analogs include modified forms of naturally and non-naturally occurring amino acids. Such modifications can include, for example, substitution or replacement of chemical groups and moieties on the amino acid or by derivitization of the amino acid. Amino acid mimetics include, for example, organic structures which exhibit functionally similar properties such as charge and charge spacing characteristic of the reference amino acid. For example, an organic structure which mimics Arginine (Arg or R) would have a positive charge moiety located in similar molecular space and having the same degree of mobility as the ε-amino group of the side chain of the naturally occurring Arg amino acid. Mimetics also include constrained structures so as to maintain optimal spacing and charge interactions of the amino acid or of the amino acid functional groups. Those skilled in the art know or

can determine what structures constitute functionally equivalent amino acid analogs and amino acid mimetics.

Specific examples of amino acid analogs and mimetics can be found described in, for example, Roberts and Vellaccio, The Peptides: Analysis, Synthesis, Biology, Eds. Gross and Meinhofer, Vol. 5, p. 341, Academic Press, Inc., New York, New York (1983), the entire volume of which is incorporated herein by reference. Other examples include peralkylated amino acids, particularly permethylated amino acids. See, for example, Combinatorial Chemistry, Eds. Wilson and Czarnik, Ch. 11, p. 235, John Wiley & Sons Inc., New York, New York (1997), the entire book of which is incorporated herein by reference. Yet other examples include amino acids whose amide portion (and, therefore, the amide backbone of the resulting peptide) has been replaced, for example, by a sugar ring, steroid, benzodiazepine or carbo cycle. See, for instance, Burger's Medicinal Chemistry and Drug Discovery, Ed. Manfred E. Wolff, Ch. 15, pp. 619-620, John Wiley & Sons Inc., New York, New York (1995), the entire book of which is incorporated herein by reference. Methods for synthesizing peptides, polypeptides, peptidomimetics and proteins are well known in the art (see, for example, U.S. Patent No. 5,420,109; M. Bodanzsky, Principles of Peptide Synthesis (1st ed. & 2d rev. ed.), Springer-Verlag, New York, New York (1984 & 1993), see Chapter 7; Stewart and Young, Solid Phase Peptide Synthesis, (2d ed.), Pierce Chemical Co., Rockford, Illinois (1984), each of which is incorporated herein by reference).

As used herein, the term "immune response" is intended to mean to a measurable or observable reaction to an antigen or immunomodulatory molecule mediated by one or more cells of the immune system. An immune response begins with an antigen or immunomodulatory molecule binding to an immune system cell and terminates with destruction of antigen and cells containing antigen or alteration in immune cell function. A reaction to an antigen or immunomodulatory molecule is mediated by many cell types, including a cell that initially binds to an antigen or immunomodulatory molecule and cells that participate in mediating an innate, humoral, cell-mediated immune response. An innate immune response involves binding of pathogen-associated molecular patterns (PAMPs) to cell surface receptors, such as toll-like receptors. Activation of toll-like receptors in response to PAMPs leads to the production of immunomodulatory molecules, such as cytokines and co-stimulatory molecules, that induce an immune response. A humoral response involves interaction of B cells with antigen and B cell differentiation into antibody-secreting cells. A cell-mediated response involves various subpopulations of T cells that recognize antigen presented on self-cells, including helper T cells that respond to antigen by producing cytokines and cytotoxic T cells that respond to antigen by developing into cytotoxic T lymphocytes, which mediate killing of altered self-cells. The term immune response includes measurable or observable reactions produced by any cell type that participates in the processes through which immune system cells are activated and antigen containing cells are destroyed. Such measurable reactions include,

for example, production of immunomodulatory molecules, migration and proliferation.

An "immunomodulatory molecule" is a molecule that alters an immune response. An immunomodulatory molecule can be, for example, a compound, such as an organic chemical; a polypeptide, such as an antibody or cytokine; a nucleic acid, such as a DNA or RNA molecule; or any other type of molecule that alters an immune response. An immunomodulatory molecule can alter an immune response by directly or indirectly altering an activity of a cell that mediates an immune response. An immunomodulatory molecule can act directly on an immune system cell, for example, by binding to a cell surface receptor and stimulating or inhibiting proliferation, differentiation, or expression, secretion or receptor binding of immune system regulatory molecules such as co-stimulatory receptors and ligands, cytokines, and chemokines. Examples of naturally occurring molecules that act directly on immune system cells to alter an immune response include PAMPs, cytokines, chemokines and growth factors. Other examples of molecules that act directly on immune system cells to alter an immune response include molecules that alter receptor functions, such as antibodies to receptors, soluble cytokine receptors, receptor agonists and antagonists, molecules that alter the production of immunomodulatory molecules, such as inhibitors of converting enzymes and molecules involved in the intracellular transport and secretion of immunomodulatory molecules.

An immunomodulatory molecule can indirectly alter the activity of a particular immune system cell by

altering the amount or activity of a molecule that regulates a cellular activity of the cell. For example, a cytokine, chemokine, or growth factor produced by an immune system cell, such as a macrophage, can stimulate or inhibit various cellular activities of B and T lymphocytes. Immune cell functions that can be stimulated or inhibited by an immunomodulatory molecule include, for example, immune cell activation, co-activation, proliferation, production of cytokines, cellular interactions and migration. An immunomodulatory molecule can therefore act on a variety of immune cell types and can alter a variety of cellular functions. An immunomodulatory flagellin peptide, polypeptide or modifications thereof used in the methods of the invention are examples of immunomodulatory molecules useful for inducing an immune response, for example, by binding to TLR5 and inducing a TLR5-mediated increase in macrophage production of TNFα, IL-1 and IL-6. The flagellin polypeptides, peptides and modifications thereof, are also useful for indirectly inducing an immune response because immunomodulatory molecules produced by a TLR5-expressing cell in response to flagellin will alter the activities of immune system cells that respond to the particular immunomodulatory molecules produced.

An immunomodulatory molecule can mediate an immune response that is specific for a target antigen or nonspecific. A specific immunomodulatory molecule alters an immune response to a particular target antigen. Examples of specific immunomodulatory molecules include monoclonal antibodies, including naked monoclonal antibodies, drug-, toxin- or radioactive

compound-conjugated monoclonal antibodies, and ADCC targeting molecules. Such immunomodulatory molecules stimulate an immune response by binding to antigens and targeting cells for destruction. An immunomodulatory molecule can be used to suppress an immune response to an antigen. For example, a tolerogenizing molecule can be used to suppress an immune response to a self-antigen.

Nonspecific immunomodulatory molecules stimulate or inhibit the immune system in a general manner through various mechanisms that can include, for example, stimulating or suppressing cellular activities of immune system cells. Nonspecific immunomodulatory molecules useful for stimulating an immune responses include, for example, agents that stimulate immune cell proliferation, immune cell activation and production of cytokines and co-stimulatory molecules. Well known immunomodulatory molecules that stimulate an immune response are, for example, interleukins, interferons, levamisole and keyhole limpet hemocyanin. Nonspecific immunomodulatory molecules useful for suppressing immune responses include, for example, agents that inhibit cytokines synthesis or processing, specific cytokine receptor blocking reagents such as soluble receptors and receptor antagonists, and cytokines that down-regulate or inhibit the production of other immunomodulatory molecules. Well known immunomodulatory molecules for suppressing an immune response include, for example, cyclosporin, rapamycin, tacrolimus, azathioprine, cyclophosphamide and methotrexate.

Immunomodulatory molecules can be contained in a mixture of molecules, including a natural or man-made

composition of molecules. Exemplary natural compositions of immunomodulatory compounds include, for example, those contained in an organism such as Bacille Calmette-Guerin (BCM) or *Corynbacterium parvum*. Exemplary man-made compositions of immunomodulatory molecules include, for example, QS-21, DETOX and incomplete Freund's adjuvant.

As used herein, the term "adjuvant" when used in reference to a vaccine, is intended to mean a substance that acts generally to accelerate, prolong, or enhance the quality of specific immune responses to a vaccine antigen. An adjuvant can advantageously reduce the number of immunizations or the amount of antigen required for protective immunization.

As used herein, the term "antigen-specific immune response" is intended to mean a reaction of one or more cells of the immune system to a particular antigen that is not substantially cross-reactive with other antigens.

As used herein, the term "antigen" is intended to mean a molecule which induces an immune response. An antigen can be a crude mixture of molecules, such as a cell, or one or more isolated molecules. Examples of crude antigens include attenuated organisms, inactivated organisms, viral particles and tumor cells. Examples of isolated antigens include a polypeptide, lipoprotein, glycoprotein, lipid, anti-idiotype antibody, toxoid, polysaccharide, capsular polysaccharide and nucleic acid. Such isolated antigens can be naturally occurring, recombinantly produced, or synthesized. Exemplary naturally occurring antigens include purified microbial

macromolecules. Exemplary recombinantly produced antigens include cloned microbial and tumor cell antigens. Exemplary synthesized antigens include synthetic peptides and nucleic acids.

As used herein, the term "vaccine" is intended to mean a compound or formulation which, when administered to an individual, stimulates an immune response against an antigen. A vaccine is useful for preventing or ameliorating a pathological condition that will respond favorably to immune response modulation. A vaccine can contain isolated or crude antigen, and can contain one or more antigens. A vaccine can contain one or more adjuvants.

As used herein, the term "immunogenic amount" is intended to mean an amount of an immunomodulatory flagellin polypeptide, peptide or modifications thereof, or combinations thereof with one or more molecules, such as an antigen or other immunomodulatory molecule, required to effect an immune response. The dosage of an immunomodulatory flagellin polypeptide, peptide, or modifications thereof, independently or in combination with one or more molecules, will depend, for example, on the pathological condition to be treated, the weight and condition of the individual and previous or concurrent therapies. The appropriate amount considered to be an immunogenic dose for a particular application of the method can be determined by those skilled in the art, using the guidance provided herein. For example, the amount can be extrapolated from *in vitro* or *in vivo* assays as described below. Those skilled in the art will understand that the condition of the patient needs to be

monitored through the course of therapy and that the amount of the composition that is administered can be adjusted according to patient response to therapy.

The term "pathologically aberrant cell" is intended to mean a cell that is altered from a normal physiological or cellular state. Such alteration can be due to changes in physiology or phenotype associated with a disease or abnormal condition of a mammalian cell or tissue. Pathologically aberrant cells include cells lacking normal control of cellular functions, such as growth, differentiation, and apoptosis, resulting in altered gene and protein expression. Cells that lack normal growth control proliferate in the absence of appropriate growth signals, resulting in damage in structure or function of surrounding tissues. Cells that lack normal differentiation undergo inappropriate phenotypic or physiological changes that do not normally characterize the cell type, resulting in damage in structure and function or surrounding tissues. Cells that lack normal apoptosis fail to undergo, or inappropriately undergo the process of cell death, resulting in damage in structure or function of surrounding tissues. Altered protein expression is an example of a phenotype change that renders such cells distinguishable from normal. For example, increased or decreased expression of a polypeptide normally expressed on a cell, expression of a mutated polypeptide and expression of a polypeptide not normally expressed on a cell are phenotypic changes that can alter a cell from normal. Examples of pathologically aberrant cells include tumor cells and degenerating cells.

As used herein, the term "pathological condition" is intended to mean a disease, abnormal condition or injury of a mammalian cell or tissue. Such pathological conditions include, for example, hyperproliferative and unregulated neoplastic cell growth, degenerative conditions, inflammatory diseases, autoimmune diseases and infectious diseases. Pathological conditions characterized by excessive or unregulated cell growth include, for example, hyperplasia, cancer, autoimmune disease and infectious disease. Hyperplastic and cancer cells proliferate in an unregulated manner, causing destruction of tissues and organs. Specific examples of hyperplasias include benign prostatic hyperplasia and endometrial hyperplasia. Specific examples of cancer include prostate, breast, ovary, lung, uterus, brain and skin cancers. Abnormal cellular growth can also result from infectious diseases in which foreign organisms cause excessive growth. For example, human papilloma viruses can cause abnormal growth of skin cells. The growth of cells infected by a pathogen is abnormal due to the alteration of the normal condition of a cell resulting from the presence of a foreign organism. Specific examples of infectious diseases include DNA and RNA viral diseases, bacterial diseases, parasitic diseases. Similarly, the growth of cells mediating autoimmune and inflammatory diseases are aberrantly regulated which results in, for example, the continued proliferation and activation of immune mechanisms with the destruction of tissues and organs. Specific examples of autoimmune diseases include, for example, rheumatoid arthritis and systemic lupus erythmatosis. Specific examples of degenerative disease include osteoarthritis and Alzheimer's disease.

By specific mention of the above categories of pathological conditions, those skilled in the art will understand that such terms include all classes and types of these pathological conditions. For example, the term cancer is intended to include all known cancers, whether characterized as malignant, benign, soft tissue or solid tumor. Similarly, the terms infectious diseases, degenerative diseases, autoimmune diseases and inflammatory diseases are intended to include all classes and types of these pathological conditions. Those skilled in the art will know the various classes and types of proliferative, infectious, autoimmune and inflammatory diseases.

As used herein the term "toll-like receptor 5" or "TLR5" is intended to mean a toll-like receptor 5 of any species, such as the murine and human polypeptides containing the amino acid sequences set forth as SEQ ID NOS:4 and 6, respectively, encoded by the nucleic acid sequence identified as SEQ ID NOS:3 and 5, respectively. A TLR5 is activated upon binding to flagellin, an immunomodulatory flagellin peptide, or modifications thereof, and other TLR5 agonists. Upon activation, a TLR5 induces a cellular response by transducing an intracellular signal that is propagated through a series of signaling molecules from the cell surface to the nucleus. For example, the intracellular domain of TLR5 recruits an adaptor protein, MyD88, which recruits the serine kinase IRAK. IRAK forms a complex with TRAF6, which then interacts with various molecules that participate in transducing the TLR signal. These molecules and other TRL5 signal transduction pathway components stimulate the activity of transcription

factors, such as fos, jun and NF-κB, and the corresponding induction of gene products of fos-, jun- and NF-κB-regulated genes, such as, for example, TNFα, IL-1 and IL-6. The activities of signaling molecules that mediate the TLR5 signal, as well as molecules produced as a result of TLR5 activation are TLR5 activities that can be observed or measured. Therefore, a TLR5 activity includes binding to a flagellin polypeptide, immunomodulatory flagellin peptide, or a modification thereof, recuitment of intracellular signaling molecules, as well as downstream events resulting from TLR5 activation, such as transcription factor activation and production of immunomodulatory molecules. A TLR5 cellular response mediates an innate immune system response in an animal because cytokines released by TLR5-expressing cells regulate other immune system cells to promote an immune response in an animal. Therefore, as used herein the term "TLR5-mediated response" is intended to mean the ability of a flagellin polypeptide, immunomodulatory peptide or modification thereof to induce a TLR5-mediated cellular response. Exemplary TLR5-mediated cellular responses include activation of transcription factors such as fos, jun and NF-κB, production of cytokines such as IL-1, IL-6 and TNFα, and the stimulation of an immune response in an animal.

A TLR5 also encompasses polypeptides containing minor modifications of a native TLR5, and fragments of a full-length native TLR5, so long as the modified polypeptide or fragment retains one or more biological activities of a native TLR5, such as the abilities to

stimulate NF-kB activity, stimulate the production of cytokines such as TNFα, IL-1, and IL-6 and stimulate an immune response in response to TLR5 binding to flagellin polypeptide, immunomodulatory peptide or modifications thereof. A modification of a TLR5 can include additions, deletions, or substitutions of amino acids, so long as a biological activity of a native TLR5 is retained. For example, a modification can serve to alter the stability or activity the polypeptide, or to facilitate its purification. Modifications of polypeptides as described above in reference to flagellin polypeptides and peptides are applicable to TLR5 polypeptides of the invention. A "fragment" of a TLR5 is intended to mean a portion of a TLR5 that retains at least about the same activity as a native TLR5.

As used herein, the term "TLR5 agonist" refers to a compound that selectively activates or increases normal signal transduction through TLR5. As used herein, the term "TLR5 antagonist" refers to a compound that selectively inhibits or decreases normal signal transduction through TLR5. A TLR5 agonist or antagonist can alter normal signal transduction through TLR5 indirectly, for example, by modifying or altering the native conformation of TLR5 or a TLR5 ligand. For therapeutic applications, a TLR5 agonist or antagonist has an EC50 of less than about 10^{-7} M, such as less than 10^{-8} M and less than 10^{-9} M, although a TRL5 agonist with a higher EC50 can be therapeutically useful. As used herein, the term "TLR5 ligand" refers to a compound that binds a TLR5 polypeptide with high affinity. A TLR5 ligand can further be an agonist or antagonist of TLR5,

as described above, or can be a compound having little or no effect on TLR5 signaling.

As used herein, the term "detectably labeled" refers to derivitization with, or conjugation to, a moiety that is detectable by an analytical or qualitative method. A detectable moiety can be, for example, a radioisotope, such as ^{14}C, ^{131}I, ^{32}P or ^{3}H, fluorochrome, ferromagnetic substance, or luminescent substance.

As used herein the term "ADCC targeting molecule" is intended to mean an antigen binding protein containing a Fc receptor binding domain capable of inducing antibody-dependent cell cytotoxicity (ADCC). An ADCC targeting molecule can also contain other domains that augment induction of ADCC. The flagellin polypeptides and peptides, immunomodulatory peptides, and modifications described herein, can be domains of an ADCC targeting molecule that augment induction of ADCC. The ADCC targeting molecule can include multiple valencies for either or both the antigen binding domain or the Fc receptor binding domain. Additionally, an ADCC targeting molecule also can have multiple different antigen binding domains combined with a single or multiple copies of an Fc receptor binding domain or combined with different Fc receptor binding domains. The antigen binding domain or domains can be derived from essentially any molecule that has selective or specific binding activity to a target antigen so long as it can be fused or attached to one or more Fc receptor binding domains while still maintaining antigen binding activity. The Fc receptor binding domain can be derived from an antibody constant region of, for example, the IgG class, including subclasses IgG1, IgG3

and IgG4. Such Fc receptor binding domains can be used in their native form or the amino acid sequence can be modified so as to enhance or optimize the Fc receptor binding or ADCC activity. Moreover, the Fc receptor binding domains can be derived from constant regions which recognize either stimulatory or inhibitory Fc receptors. The Fc receptor binding domain is located within the hinge region of an antibody constant region where the cognate receptors bound by this domain include, for example, the Fc RI, Fc RIIA and Fc RIII. Therefore, ADCC targeting molecules include, for example, antibodies selective for a target antigen and functional variants thereof as well as fusion proteins and chemical conjugates containing both an antigen binding domain and a Fc receptor binding domain in functionally active forms. ADCC targeting molecules and the use of ADCC targeting molecules in the treatment of disease are described in detail in U.S. Patent Application 09/618,176, which is incorporated herein by reference.

The term "about" when used in reference to a particular activity or measurement is intended to refer to the referenced activity or measurement as being within a range values encompassing the referenced value and within accepted standards of a credible assay within the art, or within accepted statistical variance of a credible assay within the art.

As used herein, the term "substantially" or "substantially the same" when used in reference to an amino acid sequence is intended to mean that the amino acid sequence shows a considerable degree, amount or extent of sequence identity when compared to the

reference sequence. Such considerable degree, amount or extent of identity is further considered to be significant and meaningful and therefore exhibit characteristics which are definitively recognizable or known as being derived from or related to flagellin. For example, an amino acid sequence which is substantially the same amino acid sequence as an flagellin peptide, including fragments thereof, refers to a sequence which exhibits characteristics that are definitively known or recognizable as being sufficiently related to flagellin so as to fall within the classification of flagellin sequences as defined above. Minor modifications thereof are included so long as they are recognizable as an flagellin sequence as defined above.

As used herein, the term "individual" is intended to mean any animal in which an immune response can be induced by a flagellin polypeptide, peptide or modifications thereof including a human, non-human primate, cow, pig, chicken, rabbit, ferret, rat or mouse.

An immunomodulatory flagellin polypeptide, peptide or modifications thereof can be used to induce an immune response in an individual having a pathological condition, promoting the individual's own immune system to function more effectively and thereby ameliorate the pathological condition. An individual's immune system may not recognize cancer cells and other types of pathologically aberrant cells as foreign because the particular antigens are not different enough from those of normal cells to cause an immune reaction. In addition, the immune system may recognize cancer cells, but induce a response insufficient to destroy the cancer.

By stimulating an innate immune response, immunomodulatory flagellin peptide, polypeptide or modification thereof, promote humoral and cell-mediated responses to antigens on foreign cells or pathologically aberrant cells, such as cancer cells. Administered independently or in combination with an antigen, such as a tumor antigen, a flagellin polypeptide, peptide or modification thereof, can be used to boost the immune system's recognition of cancer cells and other pathologically aberrant cells, and target such cells for destruction.

Flagellin is a pathogen-associated molecular pattern (PAMP) recognized by toll-like receptor 5 (TRL5). Toll-like receptor 5 is a member of a family of at least 10 receptors involved in mediated the innate immune response. Toll-like receptors recognize PAMPs that distinguish infectious agents from self and mediating the production of immunomodulatory molecules, such as cytokines, necessary for the development of effective adaptive immunity (Aderem, A and Ulevitch, R.J. Nature 406:782-787 (2000) and Brightbill, H.D., Immunology 101: 1-10 (2000)). Members of the toll-like receptor family recognize a variety of antigen types and can discriminate between pathogens. For example, TLR2 recognizes various fungal, Gram-positive, and mycobacterial components, TLR4 recognizes the Gram-negative product lipopolysaccharide (LPS), and TLR9 recognizes nucleic acids such as CpG repeats in bacterial DNA. TLR5 has now been identified as a receptor for bacterial flagellin.

Flagellin induces an innate immune response by binding to and activating TLR5. Activation of TLR5 by

binding to flagellin induces the production of immunomodulatory molecules, such as cytokines and co-stimulatory molecules, by a TLR5-expressing cell. For example, activation of TLR5 in macrophages results in the expression of the cytokines TNFα, IL-1 and IL-6. These cytokines directly and indirectly alter the activities of immune system cells that participate in both humoral (TH2) and cell-mediated (TH1) adaptive immune responses. In this manner, an immunomodulatory flagellin peptide, polypeptide or modification thereof, acts as an adjuvant to stimulate a general immune response.

Altering the balance of TH1- versus TH2-associated cytokines can be used to favorably alter an immune response to treat certain diseases. For example, in the use of cancer vaccines, it can be favorable to induce both TH1 and TH2 responses (Herlyn and Birebent, Ann. Med., 31:66-78, (1999)). Different sets of cytokines orchestrate TH1 and TH2 immune responses. For example, TH1 immune responses are associated with the cytokines IL-2, IFN-γ, and TNFα while TH2 immune responses are associated with the cytokines IL-4, IL-5, IL-6 and IL-10. TLR5 stimulates the production of cytokines associated with both TH1- and TH2-associated cytokines. For example, TNFα is associated with the stimulation of a TH1 type immune response (Ahlers, JD et al. J. Immunol, 158:3947-58 (1997)), and IL-6 is associated with the stimulation of a TH2 type response (Steidler, L. et al. Infect. Immun., 66:3183-9, (1998)). Therefore, an immunomodulatory flagellin peptide, polypeptide or modification thereof, can be used to advantageously elicit TH1 and TH2 type immune responses.

An immunomodulatory flagellin peptide, polypeptide or modification thereof can also be used to generally alter the particular cytokines involved in an immune response in an individual. Alterations from normal levels of cytokines are observed in many disease states. For this reason, it can be desirable to increase or decrease the amounts or activities of specific cytokines involved in particular pathological conditions. The cytokines produced in response to TLR5 activation can both stimulate and down-regulate the production of other cytokines. Therefore, an immunomodulatory flagellin peptide, polypeptide or modification thereof, or combination of a flagellin molecule with an immunomodulatory molecule or antigen can be used to alter levels of cytokines associated with a pathological condition. For example, an immunomodulatory flagellin peptide can increase TLR5-expressing macrophage production of TNFα, IL-1 and IL-6. TNFα and IL-1 generally function as pro-inflammatory cytokines. IL-6 generally functions as an anti-inflammatory cytokine and induces a variety of anti-inflammatory activities in immune system cells. For example, IL-6 stimulates the production of many anti-inflammatory anti-proteases. Those skilled in the art will be able to determine if a pathological condition in an individual could be ameliorated by inducing TLR5-stimulated cytokine production and will be able to determine appropriate combinations of flagellin and immunomodulatory molecules suitable for inducing a beneficial immune response.

The invention provides an immunomodulatory flagellin peptide comprising at least about 10 amino acids of substantially the amino acid sequence

GAVQNRFNSAIT (SEQ ID NO:2), or a modification thereof, that binds to toll-like receptor 5 (TLR5).

The flagellin peptide identified by SEQ ID NO:2 is a peptide of *S. Typhimurium1* flagellin which is encoded by the nucleic acid sequence identified by SEQ ID NO:1. A flagellin peptide of the invention also includes peptides from other bacterial species, such as *H. Pylori, V. Cholera, S. marcesens, S. flexneri, T. Pallidum, L. pneumophila, B burgdorferei, C. difficile, R. meliloti, A. tumefaciens, R. lupini, B. clarridgeiae, P. Mirabilis, B. subtilus, L. monocytogenes, P. aeruginosa* and *E. coli*, which contain amino acid sequences having 21-71% identity over the 12 amino acid sequence of SEQ ID NO:2. Thus, a flagellin peptide of the invention can have greater than about 65% identity, such as greater than about 75%, greater than about 85%, greater than about 95%, greater than about 98% identity with the peptide identified by SEQ ID NO:2.

A flagellin peptide of the invention is derived from a conserved region of a flagellin polypeptide. Conserved regions of flagellin are well known in the art and have been described, for example, in Mimori-Kiyosue, et al., J. Mol. Viol. 270:222-237, (1997). Whereas T cell receptors which mediate the adaptive immune response recognize random portions of antigen amino acid sequences, toll-like receptors recognize conserved portions of antigen amino acid sequences. Therefore, the flagellin peptides of the invention and immunomodulatory flagellin peptides used in the methods of the invention contain amino acid sequences derived from conserved regions of flagellin.

A flagellin peptide of the invention excludes a portion of flagellin described in Newton et al. (supra, 1989), which consists of an *S. meunchen* flagellin fragment containing a deletion of amino acids 207-223, portions of *E. coli* (strain K12) flagellin described in Kuwaijima et al. (supra, 1998), which consist of *E. coli* flagellin fragments containing deletions of amino acids 239-254, 259-278, 237-262, 194-379, 201-318, 218-326, 211-347, 210-299, 245-301, and 220-299, a portion of flagellin described in Samatey et al. (supra, 2000), which consists of an *S. typhimurium* flagellin fragment lacking 52 N-terminal amino acid residues and lacking 44 C-terminal amino acid residues, and portions of flagellin described in McSorley et al. (supra, 2000) which consist of *S. typhimurium* flagellin fragments having the following amino acid sequences: RSDLGAVQNRFNSAI, DLGAVQNRFNSAITN, GAVQNRFNSAITNLG AND VQNRFNSAITNLGNT.

An immunomodulatory flagellin peptide of the invention can contain a heterologous amino acid sequence that imparts structural or functional characteristics onto the flagellin peptide. For example, chimeric flagellin peptides or modifications can be used to impart a targeting function. Targeting of a flagellin peptide or modification to a particular site, such as a mucosal surface for example, confers additional therapeutic advantage of inducing an immune response at a site of pathological condition or a site favored for inducing an antigen-specific immune response, for example by a vaccine. Further, chimeric flagellin peptides can include a sequence that facilitates detection, purification or enhances immunomodulatory activity of the flagellin peptide. A flagellin peptide can be contained,

for example, in an ADCC targeting molecule used to treat a pathological condition. A flagellin peptide can augment the effectiveness of an ADCC targeting molecule by, for example, stimulating an innate immune response through TLR5, such as the induction of cytokines such as TNFα, IL-1 and IL-6. Similarly, a flagellin peptide can contain amino acid sequences of a variety of antigen polypeptides, such as those described above in reference to antigens contained in vaccines used in the methods of the invention. A chimeric flagellin peptide containing amino acid sequences of an antigen or containing an antigenic molecule such as a carbohydrate, nucleic acid, or lipid, can be used analogously to a vaccine, as described above, as well as in a vaccine formulation, to induce an immune response in an individual. As such, a chimeric flagellin peptide can be a vaccine that induces both innate and adaptive immune system responses.

An immunomodulatory flagellin peptide of the invention can be prepared by a variety of methods well-known in the art, for example, by recombinant expression systems described below, and biochemical purification methods described below, as well as by synthetic methods well known in the art. Methods for recombinant expression and purification of polypeptides in various host organisms are described, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1992) and in Ansubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1998), both of which are incorporated herein by reference. Similarly, flagellin peptide modifications can be generated using recombinant mutagenesis, such as site directed

mutagenesis and PCR mutagenesis, and expression of the flagellin peptide modification. Numerous methods of constructing, modifying, expressing and purifying peptides are known to those skilled in the art. A specific example of a method for purifying flagellin is described below in Example III. The choice of recombinant methods, expression and purification systems will be known by those skilled in the art and will depend on the user and the particular application for the immunomodulatory flagellin peptide or modification thereof.

A flagellin peptide of the invention induces an innate immune response in an individual by binding to an stimulating TLR5. Therefore, the invention provides methods for inducing an immune response in an individual having a pathological condition that can be ameliorated by immune system activity. The methods involve administering an immunomodulatory flagellin peptide or modification thereof to induce an immune response, administering a combination of an immunomodulatory flagellin peptide and an antigen to induce an antigen-specific immune response, and administering a combination of an immunomodulatory flagellin peptide and an immunomodulatory molecule to modulate an immune response. The selection of a particular method for inducing an immune response will depend on the particular pathological condition to be ameliorated or prevented in an individual. As described herein, the methods are applicable to a wide variety of pathological conditions. Those skilled in the art will be able to determine if an immune response can be beneficially modulated by administering an immunomodulatory flagellin peptide or

combination thereof with an antigen or immunomodulatory molecule.

The invention provides method of inducing an antigen-specific immune response in an individual. The method involves administering to an individual an immunogenic amount of a vaccine, comprising an antigen and an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof.

As an adjuvant in a vaccine formulation, the immunomodulatory flagellin peptides of the invention can contribute to the effectiveness of the vaccine by, for example, enhancing the immunogenicity of weaker antigens such as highly purified or recombinant antigens, reducing the amount of antigen required for an immune response, reducing the frequency of immunization required to provide protective immunity, improve the efficacy of vaccines in individuals with reduced or weakened immune responses, such as newborns, the aged, and immunocompromised individuals, and enhance the immunity at a target tissue, such as mucosal immunity, or promote cell-mediated or humoral immunity by eliciting a particular cytokine profile. An immunomodulatory flagellin peptide, polypeptide or modification thereof induces an innate immune response through activation of TLR5. The innate immune response increases the immune response to an antigen by stimulating the adaptive immune response. Therefore, a combination of an immunomodulatory flagellin peptide, polypeptide or modification thereof with one or more antigens provides

an effective vaccine for inducing an immune response in an individual.

The methods of the invention for inducing an antigen-specific immune response can be used to treat individuals having a variety of pathological conditions. For example, cancer vaccines have been used effectively for treating melanoma and breast cancers. Vaccines have been used for treatment of inflammatory diseases such as asthma (Scanga C.B and Le Gros, G., Drugs 59(6), 1217-1221 (2000)), infectious diseases of pathogenic bacteria such as *H. pylori*, pathogenic viruses such as human papilloma virus and HIV (Sutton P. and Lee, A, Aliment Pharmacol. 14:1107-1118 (2000)), protozoa, autoimmune diseases such as diabetes (von Herrath and Whitton, Ann. Med. 32:285-292 (2000)) and degenerative diseases such as Alzheimer's disease (Youngkin, S.G., Nat. Med., 7(1):18-19 (2001)). Therefore, a vaccine used in the methods of the invention for inducing an antigen-specific immune response can be administered to an individual for treatment of a variety of pathological conditions, including proliferative disease, infectious disease, inflammatory disease and degenerative disease.

A variety of antigens can be used in combination with an immunomodulatory flagellin peptide of the invention for preparing a vaccine. Microorganisms such as viruses, bacteria and parasites contain substances that are not normally present in the body. These substances can be used as antigens to produce an immune response to destroy both the antigen and cells containing the antigen, such as a bacterial cell or cancer cell.

For example, isolated or crude antigens of microbial pathogens can be used in vaccines to treat infectious disease; isolated or crude tumor cell antigens can be used in vaccines to treat cancer; isolated or crude antigens known to be associated with a pathologically aberrant cell can be used to treat a variety of diseases in which it is beneficial to target particular cells for destruction.

A variety of substances can be used as antigens in a vaccine compound or formulation. For example, attenuated and inactivated viral and bacterial pathogens, purified macromolecules, polysaccharides, toxoids, recombinant antigens, organisms containing a foreign gene from a pathogen, synthetic peptides, polynucleic acids, antibodies and tumor cells can be used to prepare a vaccine useful for treating a pathological condition. Therefore, an immunomodulatory flagellin peptide of the invention can be combined with a wide variety of antigens to produce a vaccine useful for inducing an immune response in an individual. Those skilled in the art will be able to select an antigen appropriate for treating a particular pathological condition and will know how to determine whether a crude or isolated antigen is favored in a particular vaccine formulation.

An isolated antigen can be prepared using a variety of methods well known in the art. A gene encoding any immunogenic polypeptide can be isolated and cloned, for example, in bacterial, yeast, insect, reptile or mammalian cells using recombinant methods well known in the art and described, for example in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring

Harbor Laboratory, New York (1992) and in Ansubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, MD (1998). A number of genes encoding surface antigens from viral, bacterial and protozoan pathogens have been successfully cloned, expressed and used as antigens for vaccine development. For example, the major surface antigen of hepatitis B virus, HbsAg, the β subunit of choleratoxin, the enterotoxin of *E. coli*, the circumsporozoite protein of the malaria parasite, and a glycoprotein membrane antigen from Epstein-Barr virus, as well as tumor cell antigens, have been expressed in various well known vector/host systems, purified and used in vaccines. An immunomodulatory flagellin peptide, polypeptide or modification thereof induces an innate immune response through TLR5 that can beneficially enhance an immune response to a recombinant antigen.

A pathologically aberrant cell to be used in a vaccine can be obtained from any source such as one or more individuals having a pathological condition or *ex vivo* or *in vitro* cultured cells obtained from one or more such individuals, including a specific individual to be treated with the resulting vaccine.

Those skilled in the art will be able to determine if a vaccine compound or formulation induces an innate, humoral, cell-mediated, or any combination of these types of immune response, as methods for characterizing these immune responses are well known in the art. For example, the ability of a vaccine compound or formulation to induce an innate immune response through TLR5 can be determined using methods described

herein as well as other methods. Such methods for detecting an innate immune response can be generally performed within hours of vaccine administration. The ability of a vaccine compound or formulation to induce a humoral response can be determined by measuring the titer of antigen-specific antibodies in an animal primed with the vaccine and boosted with the antigen, or determining the presence of antibodies cross-reactive with an antigen by ELISA, Western blotting or other well-known methods. Cell-mediated immune responses can be determined, for example, by measuring cytotoxic T cell response to antigen using a variety of methods well known in the art. Methods of detecting humoral and cell-medicated immune responses can be generally performed days or weeks after vaccine administration.

A combination of an antigen or immunomodulatory molecule and an immunomodulatory flagellin peptide, polypeptide or modification thereof, can be tested in a variety of preclinical toxicological and safety studies well known in the art. For example, such a combination can be evaluated in an animal model in which the antigen has been found to be immunogenic and that can be reproducibly immunized by the same route proposed for human clinical testing. A combination of an antigen or immunomodulatory molecule and an immunomodulatory flagellin peptide or modification thereof can be tested, for example, by an approach set forth by the Center for Biologics Evaluation and Research/Food and Drug Administration and National Institute of Allergy and Infectious Diseases (Goldenthal, KL et al. AID Res Hum Retroviruses, 9:S45-9 (1993)).

Those skilled in the art will know how to determine for a particular combination of antigen or immunomodulatory molecule and immunomodulatory flagellin polypeptide modification thereof, the appropriate antigen payload, route of immunization, volume of dose, purity of antigen, and vaccination regimen useful to treat a particular pathological condition in a particular animal species.

The invention provides a method of inducing a TLR5-mediated response. The method involves administering to a TLR5-containing cell an effective amount of an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof.

A TLR5-mediated response can be assessed in a cell or animal because TLR5 stimulates cellular activities that stimulate the immune response that occurs in an animal. For example, flagellin binding to TLR5 induces cellular events such as an increase in the amount or activity of cytokines, such as TNFα, IL-1 and IL-6. These cytokines in turn regulate the activities of immune system cells. Therefore a TLR5-mediated response can be determined by examining an immune responses in an animal and by observing particular immune system cell activities. Determination of immune responses in an animal is discussed below. Determination of immune system cell activities can be performed, for example, by observing or measuring the amount of activity of immunomodulatory molecules produced by specific types of immune cells. Cytokine production by macrophages is an exemplary immune cell activity that can be conveniently

measured using methods well known in the art and those described herein. A biological activity of a cytokine can also be assessed using methods well known in the art. TNFα activities include, for example, inducing the production of IL-1 and IL-6, activation of neutrophils and endothelial cells in inflammation, inducing acute phase reactants in liver, inducing fever. IL-1 activities include, for example, activating of endothelial cells in inflammation and coagulation, inducing acute phage reactants in liver, inducing fever and stimulating T cell proliferation. IL-6 activities include, for example, stimulating proliferation of mature B cells and inducing their final maturation into antibody-producing plasma cells, inducing IL-2 receptor expression, inducing acute phase reactants in liver, and co-stimulation of thymocytes *in vitro*. A regulatory effect of IL-6 is inhibition of TNFα production, providing negative feedback for limiting the acute inflammatory response (Feghali, C.A. and Wright, T.M., Frontiers in Bioscience, 2, d12-26 (1997) provides a summary of cytokine activities).

The invention provides a method of inducing an immune response in an individual having a pathological condition. The method involves administering to said individual an immunogenic amount of an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof.

As described above, an immunomodulatory flagellin peptide can be used to beneficially boost a

general immune response in an individual having a pathological condition by stimulating an innate immune response. An increased immune response can ameliorate a pathological condition as well as prevent a pathological condition in a healthy individual, or individual not having a pathological condition. Therefore, an immunomodulatory flagellin peptide can be administered prophylactically to an individual not having a pathological condition, if desired.

The invention provides another method of modulating an immune response in an individual having a pathological condition. The method involves administering to the individual a combination of an immunogenic amount of an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof, and another immunomodulatory molecule.

As described above, a combination of an immunomodulatory flagellin peptide with another immunomodulatory molecule can be used to advantageously induce or modulate an immune response. An immune response can be induced by combining an immunomodulatory flagellin peptide with another immunomodulatory molecule that induces an immune response in a general manner, such as an adjuvant, or can be combined with an immunomodulatory molecule that induces a particular alteration in an immune cell activity. Such immunomodulatory molecules are described herein.

Modulating an immune response is useful for promoting a more effective or more normal immune response in an individual having a pathological condition. As described above, alterations in normal cytokine levels are associated with various pathological conditions. An immunomodulatory flagellin peptide or combination with another immunomodulatory molecule can be used to modulate cytokine levels in an individual by inducing the production of immunomodulatory molecules, such as cytokines including TNFα, IL-1, and IL-6 through TLR5, and inducing the production of suppression of the same or different immunomodulatory molecules through the activity of the administered immunomodulatory molecule. Therefore, the immunomodulatory flagellin peptides of the invention can be combined with immunomodulatory molecules that alter an immune response by stimulating or inhibiting the cellular functions of immune system cells.

A variety of immunomodulatory molecules can be used in combination with an immunomodulatory flagellin peptide or modification thereof of the invention to alter an immune response in an individual. The type of alteration desired will determine the type of immunomodulatory molecule selected to be combined with an immunomodulatory flagellin peptide. For example, to promote an innate immune response, a immunomodulatory flagellin peptide can be combined with another immunomodulatory molecule that promotes an innate immune response, such as a PAMP or conserved region known or suspected of inducing an innate immune response. A variety of PAMPs are known to stimulate the activities of different members of the toll-like family of receptors. Such PAMPs can be combined to stimulate a particular

combination of toll-like receptors that induce a beneficial cytokine profile. For example, PAMPs can be combined to stimulate a cytokine profile that induces a TH1 or TH2 immune response.

Other types of immunomodulatory molecules that promote humoral or cell-mediated immune responses can be combined with a flagellin molecule of the invention. For example, cytokines can be administered to alter the balance of TH1 and TH2 immune responses. Those skilled in the art will know how to determine the appropriate cytokines useful for obtaining a beneficial alteration in immune response for a particular pathological condition.

Immunomodulatory molecules that target antigens and cells displaying antigens for destruction can be combined with a flagellin molecule of the invention. For example, the effectiveness of monoclonal antibodies and ADCC targeting molecules that recognize a particular antigen on an unwanted cell, such as a pathologically aberrant cell can be increased when administered with a flagellin molecule of the invention. Immunomodulatory molecules that stimulate or suppress cellular activities such as proliferation, migration, activation, interaction and differentiation can be combined with a flagellin molecule of the invention. For example, IL-2 can be used to stimulate proliferation of immune system cells, certain interferons can be used to interfere with the rapid growth of cancer cells or to interfere with angiogenesis, and ganulocyte-colony stimulating factor can be used to increase production of certain types of immune system cells and blood cells. A variety of immunostimulating and immunosupressing molecules and

modalities are well known in the art and can be used in combination with a flagellin polypeptide, peptide or modification thereof, of the invention. A flagellin molecule of the invention increases the beneficial effect of an immunomodulatory molecule by inducing TLR5-mediated production of immunomodulatory molecules that function in concert with a selected immunomodulatory molecule to produce a desired cytokine profile or cellular activity, or prime the adaptive immune response to respond to the selected immunomodulatory molecule.

The methods of the invention for using immunomodulatory flagellin peptides to induce an immune response are also applicable to a flagellin polypeptide, or a modification thereof. Accordingly, the invention provides a method of inducing an immune response in an individual, including a human, having a pathological condition. The method involves administering to the individual an immunogenic amount of an immunomodulatory flagellin polypeptide, or modification thereof, when the flagellin polypeptide induces an immune response.

An immunomodulatory flagellin peptide of the invention binds to TLR5 and stimulates a TLR5 activity. The ability of an immunomodulatory flagellin peptide or modification thereof to bind to TLR5 or stimulate a TLR5 activity can be determined using methods known in the art. Methods of determining specific binding interactions of flagellin peptides and modifications thereof with TLR5 can be determined using well known methods in the art such as methods of trapping ligand-receptor complexes using chemical cross-linking, and competitive inhibition of reagents specific for TLR5 such

as specific flagellin peptides or modifications, antibodies or other TLR-5 specific reagents.

Methods of determining TLR5 functional activities in response to an immunomodulatory flagellin peptide or modification thereof include methods described herein, in Examples I through IV, as well as methods known in the art. A variety of methods well known in the art can be used for determining transcription factor activities. For example, fos, jun, and NF-κB activation in response to TLR5 binding to a flagellin molecule can be detected by electrophoretic mobility shift assays well known in the art that detect NF-κB binding to specific polynucleic acid sequences, and promoter-reporter nucleic acid constructs such that, for example, β-lactamase, luciferase, green fluorescent protein or β-galactosidase will be expressed in response to contacting a TLR5 with a flagellin polypeptide, peptide or equivalent thereof. For example, a luciferase reporter plasmid in which luciferase protein expression is driven by one or more NF-kB binding sites can be transfected into a cell, as described in Examples I-IV. Activation of NF-kB results in activation of luciferase reporter expression, resulting in production of luciferase enzyme able to catalyze the generation of a molecule that can be detected by colorimetric, fluorescence, chemilluminescence or radiometric assay.

An amount or activity of a polypeptide, including a cytokine such as TNFα, IL-1 or IL-6, can be a read-out for activation of a TLR5 in response to binding an immunomodulatory flagellin peptide or modification

thereof. A variety of methods well known in the art can be used to measure cytokine amounts, such as, for example, flow cytometry methods, immunoassays such as ELISA and RIA, and cytokine RNA protection assays. Commercially available cytokine assay kits, such as ELISA assay formats, can be conveniently used to determine the amount of a variety of cytokines in a sample. Those skilled in the art will determine the particular cytokines to be measured when assessing an immune response in a cell or animal. For example, to determine whether a particular response is characterized as a TH1 or TH2 immune response, those skilled in the art will be able to select appropriate cytokines within the TH1 and TH2 categories, which are well known in the art.

A sample used for determining a TLR5-mediated response or immune response can include, for example, a fluid or tissue obtained from an animal, a cell obtained from an animal fluid or tissue, cultured cells including *in vitro* and *ex vivo* cultured cells, and lysates or fractions thereof and cultured cells that express TLR5.

An immune response in an animal is determined by the collective responses of the cells of the immune system. An immune response can be detected by observing various indicators of immune response in an animal. Such indicators include, for example, visible signs of inflammation of tissues, such as swelling, production of antibodies, such as levels of IgA, IgG and IgM in blood and levels of IgA in saliva, alterations in immune cell numbers, such as increased or decreased proliferation of particular immune cells, and in immune cell activities, such as production of immunomodulatory molecules and

second messenger molecules. For example, an immune response to a particular antigen can be observed in a animal using methods well known in the art such as delayed hypersensitivity skin tests. An immune response can be determined by the presence of antibodies cross reactive with an antigen, such as by ELISA and Western blotting, lymphocyte activation tests employing mitogen or antigen stimulation, mixed lymphocyte culture tests, assays for human T and B lymphocytes, flow cytometry and cell sorting to characterize populations of immune system cells obtained from an individual, soluble antigen uptake by macrophages, and tests of neutrophil functions (Stites et al. Basic and Clinical Immunology, 4th edition, Lange Medical Publications, Los Altos, CA (1982)). An immune response can also be assessed by examining amounts or activities of immune system mediators, such as cytokines and chemokines, in cells collected from fluids or tissues of animals. A variety of methods are well known in the art for qualitative and quantitative measurement of cytokine amount and bioassay of cytokine activity.

The methods of the invention for inducing an immune response can be used to treat any animal species having an immune response upon treatment with flagellin polypeptide, peptide, or modification thereof, and for which a stimulation of an immune response is desired. Such animals include avian species such as chicken, and mammalian species such as rodent, canine, feline, bovine, porcine and human subjects. Methods for using adjuvants with vaccines and vaccinating animals are well known in the art and are routinely used in laboratory animals. Those skilled in the art will be able to determine if a

particular animal species has a flagellin-stimulated TLR5-mediated innate immune response.

A vaccine to be used in the methods of the invention for inducing an immune response can be administered as a solution or suspension together with a pharmaceutically acceptable medium. Such a pharmaceutically acceptable medium can be, for example, water, phosphate buffered saline, normal saline or other physiologically buffered saline, or other solvent or vehicle such as glycol, glycerol, and oil such as olive oil or an injectable organic ester. A pharmaceutically acceptable medium can also contain liposomes or micelles, and can contain immunostimulating complexes prepared by mixing polypeptide or peptide antigens with detergent and a glycoside, such as Quil A. Further methods for preparing and administering an immunomodulatory flagellin polypeptide or peptide, or modification in a pharmaceutically acceptable medium are presented below, in reference to compounds that induce a TLR-mediated response.

The immunomodulatory flagellin polypeptides, peptides and modifications thereof used in the methods of the invention can be administered by a variety of routes to stimulate an immune response. For example, these immunomodulatory molecules can be delivered intranasally, subcutaneously, intradermally, intralymphatically, intramuscularly, intratumorally, orally, intravesically, intraperitoneally and intracerebrally. Oral administration is convenient and relatively safe. Oral vaccination protocols can be useful for inducing the state of immunological tolerance which normally occurs in

response to most soluble antigens and the proteolytic degradation of antigen preparations in the digestive tract. Nasal delivery routes may be useful for inducing both mucosal and systemic immune responses. A variety of devices are under development for convenient and effective delivery of formulations to the nasal cavity and pulmonary tissues. Those skilled in the art will know how to select appropriate delivery routes for particular formulations of flagellin polypeptides, peptides and modifications thereof.

The invention provides a screening composition consisting of a flagellin peptide of claim 1 and a TLR5. The composition is useful for identifying agonists, antagonists and ligands for TLR5. The characteristics of a flagellin peptide of claim 1 and preparation of a flagellin peptide are described herein. Similarly, the characteristics of a TLR5 polypeptide and modifications thereof that have a TLR5 activity, and methods for preparing a TLR5 polypeptide to be used in the methods of the invention are described herein. Chimeric TLR5s, such as the CD4-TLR5 described herein in Example I, are included in the screening compositions of the invention.

The screening composition of the invention includes, for example, cells, cell extracts and artificial signaling systems that contain a TLR5 polypeptide or modification thereof. The cell compositions of the invention include any cell in which TLR5 can couple to a signal transduction pathway to produce a detectable signal in response to an agonist, such as flagellin or a flagellin peptide. Such cells include insect cells such as *Drosophila* cells, yeast

cells such as *S. cerevisiae*, prokaryotic cells such as *E. coli*, amphibian cells such as *Xenopus* oocytes, and vertebrate cells such as mammalian primary cells, such as macrophages. Primary cells such as macrophages and other lymphocytes can be conveniently isolated from blood using methods well known in the art. Cells obtained from transgenic animals, such as transgenic mice that have been engineered by known methods of express recombinant TLR5 or TLR5 signal transduction components are also included in the screening compositions of the invention. Cell lines prepared from any of theses cell types, such as S2, CHO, NIH-3T3, 293 and HeLa cells are also included in a screening composition of the invention.

The screening compositions of the invention can include crude or partially purified lysates or extracts of the cell compositions of the invention, and reconstituted signaling systems. Artificial signaling systems include, for example, natural or artificial lipid bilayers, such as a liposome or micelle, which promote an active conformation of a TLR5. The compositions can further contain cellular fractions or isolated components necessary for producing and detecting the desired predetermined signal.

The invention provides a method of screening for a TLR5 ligand, agonist or antagonist. The method involves, (a) contacting a TLR5 with a candidate compound in the presence of a flagellin polypeptide or immunomodulatory flagellin peptide under conditions wherein binding of the flagellin polypeptide or immunomodulatory flagellin peptide to the TLR5 produces a predetermined signal; (b) determining the production of

the predetermined signal in the presence of the candidate compound; and (c) comparing the predetermined signal in the presence of the candidate compound with a predetermined signal in the absence of the candidate compound, wherein a difference between the predetermined signals in the presence and absence of the candidate compound indicates that the compound is a TLR5 ligand, agonist or antagonist.

TLR5 can produce a variety of predetermined signals useful in the methods of the invention for identifying a TLR5 ligand, agonist or antagonist. TLR5 has an extracellular domain that participates in ligand recognition and intracellular domain that contain a conserved region called the Toll/IL-1R homology (TIR) domain that, upon activation, recruits an adaptor protein, MyD88. Through an amino terminal death domain, MyD88 recruits the serine kinase IRAK to propagate a pro-inflammatory signal through binding to TRAF6, which then binds to other molecules that participate in the TLR5 signaling cascade. Immunomodulatory flagellin peptides and modifications binding to TLR5 induces signal transduction events which result in, for example, stimulating NF-κB activity and inducing production of gene products of NF-kB-regulated genes, such as TNFα, IL-1 and IL-6, as well as stimulating AP-1 transcription factors fos and jun. Therefore, a predetermined signal can include a signal produced by an immunomodulatory flagellin polypeptide or peptide or modification binding to TLR5, a signal produced by a TLR5 intracellular signal transduction even, such as kinase or phosphatase activity or protein-protein interactions, by activation of fos,

jun or NF-κB, and by an amount or activity of a fos-, jun- or NF-κB-regulated gene or gene product, such as TNFα, IL-1 and IL-6.

A variety of low- and high-throughput assays suitable for detecting selective binding interactions between a receptor and a ligand are known in the art. Both direct and competitive assays can be performed, including, for example, fluorescence correlation spectroscopy (FCS) and scintillation proximity assays (SAP) reviewed in Major, J. Receptor and Signal Transduction Res. 15:595-607 (1995); and in Sterrer et al., J. Receptor and Signal Transduction Res. 17:511-520 (1997)). Other assays for detecting binding interactions include, for example, ELISA assays, FACS analysis, and affinity separation methods. Such assays can involve labeling a TLR5 ligand, such as flagellin or a flagellin peptide, with a detectable moiety such as a radiolabel, fluorochrome, ferromagnetic substance, or luminescent substance. A detectably labeled flagellin polypeptide or peptide can be prepared using methods well known in the art. Receptor binding assays, including high-throughput automated binding assays, and methods of determining binding affinity from such assays, are well known in the art, and any suitable direct or competitive binding assay can be used. Exemplary high-throughput receptor binding assays are described, for example, in Mellentin-Micelotti et al., Anal. Biochem. 272:P182-190 (1999); Zuck et al., Proc. Natl. Acad. Sci. USA 96:11122-11127 (1999); and Zhang et al., Anal. Biochem. 268;134-142 (1999).

A variety of methods well known in the art can be used to detect activation of transcription factors, such as NF-κB, in low- or high-throughput formats. The methods described herein and in the Examples can be adapted to formats suitable for candidate compound screening.

A variety of low- and high-throughput assays suitable for detecting amounts and activities of polypeptides such as cytokines are known in the art. Methods for detecting polypeptides, include, for example, flow cytometric measurements as described herein, immunodetection methods such as radioimmune assay (RIA), ELISA, immunoprecipitation and Western blotting. Assay of the activity of a cytokine include function bioassays and detection of amounts of polypeptides regulated by a particular cytokine. Those skilled in the art can determine an appropriate method for detecting an activity of a particular cytokine.

Suitable conditions under which TLR5 produces a predetermined signal in response to a flagellin polypeptide, peptide or modification can be determined by those skilled in the art, and will depend on the particular predetermined signal selected. Exemplary conditions for determining the production of a predetermined signal are provided herein in Examples I-IV. Any known or predicted TLR5-mediated cellular event, such as elicitation of second messengers, induction of gene expression or altered cellular proliferation, differentiation or viability can be a predetermined signal that is an indication of activation of signal transduction through TLR5.

Assays for detecting a predetermined signal produced by binding of flagellin or flagellin peptide to TLR5 can be performed, for example, with whole cells that express TLR5, membrane fractions, or artificial systems, as described herein, or with isolated TLR5 polypeptide, either in solution, in an artificial membrane, or bound to a solid support.

A method of identifying TLR5 agonists and antagonists can be performed either in the presence of a predetermined concentration of a known TLR5 agonist, such as flagellin, flagellin peptide, or modifications thereof, or in the absence of agonist. The agonist can be added either prior to, simultaneously with, or after, addition of the test compound. When present, the agonist concentration is preferably within 10-fold of its EC50 under the assay conditions to allow the identification of a compound that competes with a known agonist for signaling through TLR5, or indirectly augments signaling through the receptor. Likewise, a compound that reduces binding between a known agonist and its receptor, or indirectly decreases signaling through the receptor, can also be identified.

The method of screening to identify a ligand, agonist or antagonist of TLR5 involve testing a candidate compound. A candidate compound can be any substance, molecule, compound, mixture of molecules or compounds, or any other composition. The candidate compounds can be small molecules or macromolecules, such as biological polymers, including proteins, polysaccharides and nucleic acids. Sources of candidate compounds which can be

screened for a ligand, agonist or antagonist of TLR5 include, for example, libraries of small molecules, peptides and polypeptides.

Additionally, candidate compounds can be preselected based on a variety of criteria. For example, suitable candidate compounds can be selected as having known ligand, agonist or antagonist activity. Alternatively, candidate compounds can be selected randomly. Candidate compounds can be administered to the reaction system at a single concentration or, alternatively, at a range of concentrations to determine, for example, an EC50 or IC50 of a candidate compound.

The method of screening for TLR5 ligands, agonists or antagonists can involve groups or libraries of compounds. Methods for preparing large libraries of compounds, including simple or complex organic molecules, carbohydrates, peptides, peptidomimetics, polypeptides, nucleic acids, antibodies, and the like, are well known in the art. Libraries containing large numbers of natural and synthetic compounds can be obtained from commercial sources.

The number of different candidate compounds to examine using the methods of the invention will depend on the application of the method. It is generally understood that the larger the number of candidate compounds, the greater the likelihood of identifying a compound having the desired activity in a screening assay. Large numbers of compounds can be processed in a high-throughput automated format.

The TLR5 agonists, antagonists and ligands identified using the methods and compositions described herein, are potential therapeutic compounds that can be administered to an individual, such as a human or other mammal, in an effective amount to increase or decrease signaling through TLR5, for example, to alter an immune response or treat a TLR5-associated condition. Such compounds can be used analogously to immunomodulatory compounds useful for augmenting and altering an immune response, as described above. For example, a compound can be used to induce a general immune response and to induce a specific immune response in the presence of an antigen and to alter the level of a particular cytokine in an individual having a pathological condition.

The TLR5 agonists and antagonists, immunomodulatory flagellin peptides, polypeptides and modifications thereof, are useful for ameliorating, or reducing the severity of a pathological condition. Reduction in severity includes, for example, an arrest or decrease in clinical symptoms, physiological indicators, biochemical markers or metabolic indicators of disease. Those skilled in the art will know, or will be able to determine the appropriate clinical symptoms, physiological indicators, biochemical markers or metabolic indicators to observe for a particular pathological condition. To prevent a disease means to preclude the occurrence of a disease or restoring a diseased individual to their state of health prior to disease.

In addition to applications described herein for agonists and antagonists, a TLR5 ligand can be used,

for example, to specifically target a diagnostic moiety to cells and tissues that express TLR5, such as monocytes, immature dendritic cells, epithelial cells, and other cells involved in an immune response. Thus, a TLR5 ligand can be labeled with a detectable moiety, such as a radiolabel, fluorochrome, ferromagnetic substance, or luminescent substance, and used to detect normal or abnormal expression of TLR5 polypeptide in an isolated sample or *in vivo* diagnostic imaging procedures.

A heterologous amino acid sequence can be advantageously used to provide a tag for detection or purification or to impart an activity to a reference polypeptide or peptide, such as an enzyme activity, a biological activity, an immunological activity or stability. An immunomodulatory flagellin peptide, polypeptide or modification thereof, or TLR5 polypeptide can contain a heterologous amino acid sequence, or amino acid sequence not present in the native amino acid sequence of a reference polypeptide or peptide and not represented by a modification of a reference polypeptide or peptide. A heterologous amino acid sequence can be of any size in relation to the reference amino acid sequence. A TLR5 polypeptide containing the heterologous sequence of CD4 is a specific example of such a modification and is described further in Example I. The described CD4-TLR5 chimera is identified by the amino acid sequence of SEQ ID NO:8, encoded by the nucleic acid sequence of SEQ ID NO:7. A chimeric TLR5 can be prepared using cloning methods well known in the art. For example, a chimeric polypeptide can be produced by amplifying by PCR a nucleotide sequence encoding a portion of a selected polypeptide using sequence specific

primers. Primers useful for amplifying a TLR5 include, for example, huTLR5-A6: TTAAAGTGGTACCAGTTCTCCCTTTTCATTGTATGCACT and huTLR5DNS: CGGGATCCCGTTAGGAGATGGTTGCTACAGTTTGC. A portion of a TLR5 nucleotide sequence, such as a sequence amplified using such primers can be fused to a nucleotide sequence encoding a heterologous amino acid sequence. A variety of methods for generating nucleic acid sequences encoding chimeric polypeptides are well known to those skilled in the art.

The polypeptides and peptides described herein, including immunomodulatory flagellin peptides, flagellin polypeptide, TLR5 polypeptides and fragments thereof can be prepared using a variety of protein expression systems well known in the art, including prokaryotic and eukaryotic expression systems. Prokaryotic expression systems are advantageous due to their ease in manipulation, low complexity growth media, low cost of growth media, rapid growth rates and relatively high yields. Well known prokaryotic expression systems include, for example, *E. coli* bacterial expression systems based on bacteriophage T7 RNA polymerase, the *trc* promoter, the araB promoter and bacillus expression. Eukaryotic expression systems are advantageous because expressed polypeptides can contain eukaryotic post-translational modifications such as O-linked glycosylation, phosphorylation and acetylation and can have improved protein folding. Well known eukaryotic expression systems include, for example, expression in yeast, such as *Pichia pastoris* and *Pichia methanolica*, expression in insect systems such as the Drosophila S2 system and baculovirus expression systems and expression

in mammalian cells using adenoviral vectors and cytomegalovirus promoter-containing vectors.

An immunomodulatory flagellin peptide, polypeptide, TLR5 or fragments thereof can be purified using a variety of methods of protein purification well known in the art. Biochemical purification can include, for example, steps such as solubilization of the polypeptide or peptide-expressing cell, isolation of the desired subcellular fractions, chromatography, such as ion exchange, size, or affinity-based chromatographies, electrophoresis, and immunoaffinity procedures. Other well-known methods are described in Deutscher et al., Guide to Protein Purification: Methods in Enzymology Vol. 182, (Academic Press, (1990)). An exemplary method for purifying a flagellin peptide is provided in Example III. The methods and conditions for biochemical purification of a polypeptide of the invention can be chosen by those skilled in the art, and the purification monitored, for example, by staining SDS-PAGE gels containing protein samples, by immunodetection methods such as Western blotting and ELISA, and by functional assay of immunogenic activity of flagellin or a TLR5 activity of TLR5.

An immunomodulatory flagellin peptide, polypeptide, TLR5 or fragments thereof can be modified, for example, to increase polypeptide stability, alter an activity, facilitate detection or purification, or render the enzyme better suited for a particular application, such as by altering substrate specificity. Computer programs known in the art can be used to determine which amino acid residues of a immunomodulatory flagellin

peptide, flagellin polypeptide or TLR5 can be modified as described above without abolishing a corresponding activity (see, for example, Eroshkin et al., Comput. Appl. Biosci. 9:491-497 (1993)). In addition, structural and sequence information can be used to determine the amino acid residues important for activity. For example, a comparisons of flagellin amino acid sequences, such as that shown in Figure 7 can provide guidance in determining amino acid residues that can be altered without abolishing flagellin or flagellin peptide activity by indicating amino acid residues that are conserved across species. Conserved regions of flagellin are well known in the art and have been described, for example, in Mimori-Kiyosue, et al., J. Mol. Viol. 270:222-237, (1997). A crystal structure of flagellin can also provide guidance for making flagellin modifications (Samatey et al. Nature, 410:331-337 (2001)). Similarly, amino acid sequence comparisons between the disclosed murine TLR5, TLR5s of other species, and other toll-like receptor family members can provide guidance for determining amino acid residues important for activity.

An isolated TLR5 is a TLR5 removed from one or more components with which it is naturally associated. Therefore, an isolated TLR5 can be a cell lysate, cell fraction, such as a membrane fraction, or a purified TLR5 polypeptide. An isolated TLR5 can include a liposome or other compound or matrix that stabilizes or promotes an active conformation of the receptor.

For treating or reducing the severity of a pathological condition a TLR5 agonist or antagonist,

immunomodulatory flagellin peptide, polypeptide or modification thereof, including a vaccine, can be formulated and administered in a manner and in an amount appropriate for the condition to be treated; the weight, gender, age and health of the individual; the biochemical nature, bioactivity, bioavailability and side effects of the particular compound; and in a manner compatible with concurrent treatment regimens. An appropriate amount and formulation for a particular therapeutic application in humans can be extrapolated based on the activity of the compound in recognized animal models of the particular disorder.

Animal models of aberrantly proliferative diseases can be used to assess a formulation of compound, including a vaccine or adjuvant containing an immunomodulatory flagellin peptide, polypeptide or modification thereof, for an amount sufficient to induce an immune response or ameliorate disease symptoms. Animal models of such pathological conditions well known in the art which are reliable predictors of treatments in human individuals for include, for example, animal models for tumor growth and metastasis, infectious diseases and autoimmune disease.

There are numerous animal tumor models predictive of therapeutic treatment which are well known in the art. These models generally include the inoculation or implantation of a laboratory animal with heterologous tumor cells followed by simultaneous or subsequent administration of a therapeutic treatment. The efficacy of the treatment is determined by measuring the extent of tumor growth or metastasis. Measurement of

clinical or physiological indicators can alternatively or additionally be assessed as an indicator of treatment efficacy. Exemplary animal tumor models can be found described in, for example, Brugge et al., Origins of Human Cancer, Cold Spring Harbor Laboratory Press, Plain View, New York, (1991).

Similarly, animal models predictive for infectious disease also follow a similar approach. Briefly, laboratory animals are inoculated with an infectious agent and the progression of the infection is monitored by, for example, clinical symptoms, growth culture of the agent from an infected tissue sample or biopsy in the presence or absence of the therapeutic treatment. The reduction in severity of the diagnostic indicator is indicative of the efficacy of the treatment. A variety of animal models for infectious diseases are well known to those skilled in the art.

One animal model predictive for autoimmune diseases is Experimental allergic encephalomyelitis (EAE), also called experimental autoimmune encephalomyelitis. Although originally characterized as a model for neurological autoimmune disease such as human multiple sclerosis, the use of this model to predict treatments of other autoimmune diseases has been widely accepted. EAE is induced in susceptible animals by active immunization with myelin basic protein (MPB) or by passive transfer of MBP-specific T helper lymphocytes. Progression of the disease is characterized by chronic relapsing paralysis and central nervous system demyelination, which can be monitored by observation or by immunological determinants such as delayed-type

hypersensitivity (DTH; a measure of cell mediated immunity) response to the immunogen. Efficacy of a therapeutic treatment is compared to progression of the disease in the absence of treatment. A reduction in severity of EAE symptoms or immunological determinants in treated animals is indicative of the efficacy of the therapeutic treatment. For a review of autoimmune disease models see, for example, Urban et al., *Cell*, 54:577-592 (1988); Brostoff et al., *Immunol. Ser.* 59:203-218 (1993) and U.S. Patent Nos. 5,614,192 and 5,612,035.

A growing number of human diseases have been classified as autoimmune and include, for example, rheumatoid arthritis, myasthenia gravis, multiple sclerosis, psoriasis, systemic lupus erythmatosis, autoimmune thyroiditis, Graves' disease, inflammatory bowel disease, autoimmune uveoretinitis, polymyositis and diabetes. Animal models for many of these have been developed and can be employed analogously as the EAE model described above predictive assessment of therapeutic treatments using the compounds, vaccines and adjuvants in the methods of the invention.

Other reliable and predictive animal models are well known in the art and similarly can be used to assess a compound formulation, including vaccine and adjuvant formulations containing an immunomodulatory flagellin peptide, polypeptide or modification thereof.

The total amount of a compound including an immunomodulatory flagellin peptide, polypeptide or modification thereof, that modulates a TLR5-mediated immune response can be administered as a single dose or

by infusion over a relatively short period of time, or can be administered in multiple doses administered over a more prolonged period of time. Additionally, a compound can be administered in a slow-release matrix, which can be implanted for systemic delivery at or near the site of the target tissue.

A compound that modulates a TLR5-mediated immune response can be administered to an individual using a variety of methods known in the art including, for example, intravenously, intramuscularly, subcutaneously, intraorbitally, intracapsularly, intraperitoneally, intracisternally, intra-articularly, intracerebrally, orally, intravaginally, rectally, topically, intranasally, or transdermally.

A compound that modulates a TLR5-mediated immune response can be administered to a subject as a pharmaceutical composition comprising the compound and a pharmaceutically acceptable carrier. The choice of pharmaceutically acceptable carrier depends on the route of administration of the compound and on its particular physical and chemical characteristics. Pharmaceutically acceptable carriers are well known in the art and include sterile aqueous solvents such as physiologically buffered saline, and other solvents or vehicles such as glycols, glycerol, oils such as olive oil and injectable organic esters. A pharmaceutically acceptable carrier can further contain physiologically acceptable compounds that stabilize the compound, increase its solubility, or increase its absorption. Such physiologically acceptable compounds include carbohydrates such as glucose, sucrose or dextrans; antioxidants, such as ascorbic acid or

glutathione; chelating agents; and low molecular weight proteins. As described above in reference to vaccines, such routes of administration are also applicable to administration of an immunomodulatory flagellin peptide, polypeptide or modification thereof.

In addition, a formulation of a compound that modulates a TLR5-mediated immune response can be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the compound is released systemically over time. Osmotic minipumps also can be used to provide controlled delivery of specific concentrations of a compound through cannulae to the site of interest, such as directly into a tumor growth or other site of a pathology involving a perturbation state. The biodegradable polymers and their use are described, for example, in detail in Brem et al., J. Neurosurg. 74:441-446 (1991). These methods, in addition to those described above in reference to vaccines, are applicable to administering an immunomodulatory flagellin peptide, polypeptide or modification thereof to induce an immune response.

The methods of treating a pathological condition additionally can be practiced in conjunction with other therapies. For example, for treating cancer, the methods of the invention can be practiced prior to, during, or subsequent to conventional cancer treatments such as surgery, chemotherapy, including administration of cytokines and growth factors, radiation or other methods known in the art. Similarly, for treating

pathological conditions which include infectious disease, the methods of the invention can be practiced prior to, during, or subsequent to conventional treatments, such as antibiotic administration, against infectious agents or other methods known in the art. Treatment of pathological conditions of autoimmune disorders also can be accomplished by combining the methods of the invention for inducing an immune response with conventional treatments for the particular autoimmune diseases. Conventional treatments include, for example, chemotherapy, steroid therapy, insulin and other growth factor and cytokine therapy, passive immunity and inhibitors of T cell receptor binding. The methods of the invention can be administered in conjunction with these or other methods known in the art and at various times prior, during or subsequent to initiation of conventional treatments. For a description of treatments for pathological conditions characterized by aberrant cell growth see, for example, The Merck Manual, Sixteenth Ed, (Berkow, R., Editor) Rahway, N.J., 1992.

As described above, administration of a compound, immunomodulatory flagellin peptide, flagellin polypeptide or modification thereof can be, for example, simultaneous with or delivered in alternative administrations with the conventional therapy, including multiple administrations. Simultaneous administration can be, for example, together in the same formulation or in different formulations delivered at about the same time or immediately in sequence. Alternating administrations can be, for example, delivering an immunomodulatory flagellin peptide or polypeptide formulation and a conventional therapeutic treatment in

temporally separate administrations. As described previously, the temporally separate administrations of a compound, immunomodulatory flagellin peptide, polypeptide or modification thereof, and conventional therapy can similarly use different modes of delivery and routes.

The invention provides a method of using a signal produced in response to flagellin binding to TLR5 to detect bacterial contamination in a sample. The method can be used to detect picogram amounts of flagellin in a sample.

Food-born diseases resulting from the presence of harmful bacteria account for 325,000 hospitalizations and 5,000 deaths each year in the United States (National Institutes of Health, Foodborne Diseases NIAID Fact Sheet). The U.S. Centers for Disease Control and Prevention (CDC) estimates that 1.4 million people in the United States are infected each year with *Salmonella*. Other bacterial pathogens that cause pathological conditions characterized by symptoms ranging from intestinal discomfort to severe dehydration, bloody diarrhea and even death, include enterohemorrhagic *E. coli*, such as strains designated O157:H7 and O26:H11, *Campylobacter* strains such as *C. jejuni*, and *Shigella* strains such as *S. flexneri*.

All of these bacterial strains are flagellated, and therefore express flagellin polypeptides. For example, the amino acid sequences of flagellins from *Salmonella, E. coli, Campylobacter, Shigella* strains are shown in Figure 7. The methods of the invention for detecting flagellin polypeptides contained in samples

suspected of bacterial contamination can be applied to quality assurance protocols for preparation of foods and numerous other applications.

The invention also provides a bioassay for detecting bacterial contamination in a sample. The method involves, (a) contacting the sample with a TLR5 under conditions wherein binding of a flagellin polypeptide or fragment thereof in the sample to the TLR5 produces a predetermined signal, (b) determining the production of the predetermined signal in the presence and absence of the sample, and (c) comparing the predetermined signal in the presence of the sample with a predetermined signal in the absence of the sample, wherein a difference between the predetermined signals in the presence and absence of the sample indicates that the sample contains flagellin.

The methods of the invention for detecting bacterial contamination are based on the finding disclosed herein that flagellin is a ligand for TLR5. Therefore, a flagellin molecule in a sample can bind to a TLR5 and elicit the production of a predetermined signal. A predetermined signal produced by TLR5 in a particular assay system is compared in the presence and absence of a sample known or suspected of containing a bacterial contaminant. A sample known to be free of flagellin can be used as a negative control, while a sample containing a known concentration of flagellin, flagella or bacteria having flagella can be used as a positive control.

A sample to be tested for the presence of flagellin can be any material that is suspected of being

contaminated with a gram-positive or gram-negative flagellated bacterium. For example, the method for determining the presence of flagellin can be performed using a sample of a biological fluid, cell, tissue, organ or portion thereof, such as a sample of a tissue to be used for preparing a product, a product for human or animal consumption, such as a food or pharmaceutical preparation, and a product for external application or administration by any route to an animal.

A variety of predetermined signals produced by a TLR5, as discussed above and in the Examples herein, can be used to detect the binding and activation of a TLR5 by a flagellin molecule present in a sample. A variety of methods known in the art, including those described herein can be used to detect a predetermined signal produced by a TLR5.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Constitutively Active TLR5 Activates NF-κB and TNFα Production

This example shows activation of NF-κB and TNFα production in CHO cells in response to constitutively active TLR5.

To determine if TLR5 activates NF-κB and TNFA-α production, the activity of a constitutively active form of TLR5 was examined in CHO cells. Constitutively active forms of TLR4 and TLR5 were generated by fusing the extracellular domain of CD4 to the transmembrane and TIR domain of TLR4 or TLR5 (Medzihitov, R. et al. Nature 388, 394-7 (1997); Ozinsky, A. et al., Proc. Natl. Acad. Sci. 97, 13766-13881 (2000)). CD4-TLR5 was constructed by fusing the murine CD4 extracellular domain (amino acids 1-391) to the putative transmembrane and cytoplasmic domains of human TLR5 (amino acids 639-859) and cloning into pEF6-TOPO (pEF6-mCD4-hTLR5). These chimeras, referred to as CD4-TLR4 and CD4-TLR5 were expressed in CHO cells.

For determining NF-κB activity in response to TLR5, CHO cells were transiently transfected with expression vectors for CD4-TLR4, CD4-TLR5, or empty expression vector (control) together with an NF-κB luciferase reporter. NF-κB-induced luciferase activity was measured. CHO cells (CHO-K1) were obtained from ATCC (no. CRL.-9618) and grown in Ham's F-12 medium supplemented with 10% FBS, L-glutamine, penicillin, and

streptomycin. CHO cells were transfected by electroporation as described previously (Underhill, D. M. et al., Nature, 401,811-5 (1999)), with 1 μg of the indicated TLR expression vector, 1 μg of ELAM-firefly luciferase, 0.1 μg of TK-renilla luciferase (Promega). Cells were plated on 96-well plates at 100,000 cells/well, and incubated overnight at 37°C, 5% CO_2. Firefly and renilla luciferase activities were measured using the Dual Luciferase Assay System (Promega, Madison, WI). Luciferase activity is expressed as a ratio of NF-κB-dependent ELAM-firefly luciferase activity divided by control thymidine kinase-renilla luciferase activity (relative luciferase units).

For determining TNFα production in response to TLR5, RAW-TTIO Macrophage cells were transfected with a CD4-TLR5 expression vector, and the production of TNFA-α was measured by flow cytometry, as described previously (Ozinsky, A. et al. Proc. Natl. Acad. Sci. 97, 13766-13771 (2000)). Transfections were performed by electroporation using 10 μg of pEF6-mCD4-hTLR5, and 18 hours later the cells were incubated with 5 μg/ml of brefeldin A for 4 hours to accumulate intracellular pools of newly synthesized TNFA-α. Cells were fixed, permeabilized, stained for the expression of CD4 (anti-CD4-FITC, Pharmingen) and TNFA-α (anti-murine TNFA-α-PE, Pharmingen), and analyzed on a FACscan (Beckton-Dickenson). FACS data were analyzed with WinMDI (Joseph Trotter, Scripps Research Institute, La Jolla, CA). Cells were gated to exclude dead cells and for expression of CD4.

Figure 1 shows that expression of CD4-TLR5 induced NF-kB activation-mediated luciferase production in CHO cells (Fig. 1a) and TNFA-α production in mouse macrophages (Fig. 1 b). In Figure 1b, the dotted line indicates TNFA-α produced in cells not expressing CD4-TLR5, and the solid line indicates TNFA-α produced in cells expressing CD4-TLR5.

Thus, homo-oligomerization of the TLR5 signaling domain induces a cellular signal characterized by the induction of NF-κB activity and production of TNFα.

EXAMPLE II

Bacterial Culture Supernatants Contain TLR5-Stimulating Activity

This Example shows that bacterial culture supernatants contain TLR5-stimulating activity.

CHO cells expressing human TLR5 and a luciferase-linked reporter were used to screen for PAMPs recognized by the receptor. PAMPS tested included LPS, lipopeptide, yeast, and extracts from *E.coli*, *Pseudomonas*, and *Listeria*. CHO cells were transiently transfected with TLR2, TLR5, or empty expression vectors together with a NF-κB luciferase reporter. The cells were treated with 100 ng/ml LPS, 100 ng/ml lipopeptide, 10^7 yeast particles/ml, or untreated (control), and luciferase activity was measured. The cells were treated with 67 μg/ml of supernatant from the indicated saturated

bacterial cultures, or LB alone (control), and the luciferase activity was measured. Data are representative of 3 independent experiments.

Human TLR5 and TLR2 were generated by PCR from cDNA derived from human peripheral blood mononuclear cells and cloned into pEF6-TOPO (Invitrogen) (pEF6-hTLR5 and pEF6-hTLR2). Murine TLR5 was generated by PCR using cDNA derived from RAW-TTlO cells and cloned into pEF6 (pEF6- mTLR5).

For luciferase assays, CHO cells were transfected by electroporation as described above, with 1 μg of the indicated TLR expression vector, 1 μg of ELAM-firefly luciferase, 0.1 μg of TK-renilla luciferase (Promega, Madison, WI). The medium was replaced with medium containing the stimuli at the indicated concentration/dilution. Bacterial lipopeptide and PAM_3CSK_4, were obtained from Roche, LPS (*Salmonella minnesota* R595) was from List, and yeast particles (zymosan) were from Molecular Probes (Eugene, OR). Cells were stimulated for 5 hours at 37°C, and firefly and renilla luciferase activities were measured using the Dual Luciferase Assay System (Promega).

For preparation of bacterial supernatants, bacteria were grown either in Luria broth (LB) (*Escherichia coli* TOP 10 (Invitrogen), *Salmonella minnesota* (ATCC#49284), mutant *Salmonella typhimurium* (TH4778 *fliB- fliC+*), TH2795 (*fliB- fliC-*),(Dr. Kelly Hughes, University of Washington), or grown in trypticase soy broth (TSB) (*Listeria monocytogenes* (10403, gift of

Dr. Daniel Portnoy, UCSF), *Listeria innocua* (ATCC#33090), *Bacillus subtilis*, and *Pseudomonas aeruginosa* (Susan R. Swanzy, University of Washington)). Bacteria were grown to saturation (about 16 hours, 37°C with vigorous aeration). The bacterial culture supernatants were centrifuged for 30 minutes at 2000 x g, filtered (0.2 μM), and stored at 4°C prior to use. For *flaA* transfections, *E.coli* TOP10 containing pTrcHis2-*flaA* or pTrcHis2-*flaA*rev were selected from bacterial plates and grown to OD_{600} of 0.6 in LB with 100 ug/ml ampicillin and 1% w/v glucose. The bacteria were centrifuged for 30 minutes at 2000 x g, and split into two LB cultures, one containing 100 μg/ml ampicillin and 1% w/v glucose (to repress *flaA*) and the other containing 100 μg/ml ampicillin and 1 mM IPTG (to induce *flaA*). Samples were taken at 4 hours after induction, centrifuged 5 min at 10,000 x g, and the supernatants stored at 4°C before use.

TLR5 did not respond to any of the PAMPs known to stimulate TLR pathways, such as LPS, lipopeptide, yeast cell wall, or peptidoglycan, while CHO cells transfected with TLR2 were stimulated by lipopeptide, yeast cell wall, and peptidoglycan (Fig. 2a). However, TLR5-stimulating activity was detected in culture supernatants of a variety of Gram-positive and Gram-negative bacteria (Fig. 2b). The TLR5-stimulating activity of Gram-positive bacteria was not enhanced by co-expression of CD14. Interestingly, the TOP10 strain of *E. coli* had very little TLR5 activity (Fig. 2b), and was used in subsequent reconstitution experiments (see below). Experiments using murine TLR5 yielded similar results.

Thus, the activity of TLR5 was stimulated by a component of bacterial culture supernatants, but not by PAMPs known to stimulate other toll like receptor family members.

EXAMPLE III

Purification of TLR5-Stimulating Activity from *L. monocytogenes* Culture Supernatant

This Example shows the purification of TLR5-simulating activity from *L. monocytogenes* culture supernatant.

The biological activity recognized by TLR5 was determined to be TCA precipitable, phenol soluble, and sensitive to proteinase K and trypsin digestion. To identify the bacterial components that stimulate TLR5, the supernatant from a saturated *L. monocytogenes* culture was concentrated, fractionated by reverse-phase chromatography, and each fraction was assessed for TLR5-stimulating activity in CHO cells (Fig. 3a).

For assessing the TLR-stimulating activity of FPLC fractions, CHO cells were transfected as described in Example I with the addition of 0.1 μg of pNeo/Tak (Underhill et al., Nature 401, 811-5 (1999)), and stable populations of cells expressing the indicated TLR with the luciferase reporters were selected in 100 μg/ml G418. These cells were plated on 96-well plates at 100,000 cells/well and incubated overnight.

For the purification of the TLR5-stimulating activity, saturated *L. monocytogenes* culture (200 ml of TSB) was centrifuged, and the supernatant was enriched for molecules larger than 30 kDa by ultrafiltration (Ultrafree-15 filter unit with Biomax-30 membrane, Millipore). The buffer was changed to 100 mM Tris pH 7.5, and the volume was adjusted to 5 ml. The sample was loaded onto a HR5/10 reverse-phase chromatography column (AP Biotech) and run at 0.3 ml/min. Reverse-phase chromatography was performed with the indicated elution profile using the following buffers: (A) initial buffer, 0.1% TFA in water, (B) final buffer, 0.1% TFA in acetonitrile. Fractions were collected at 3-minute intervals. FPLC fractions (50µl) were separated on a 10% SDS-PAGE gel.

As shown in Figure 3a, CHO cells expressing an NF-κB luciferase reporter and TLR5 were stimulated with reverse-phase FPLC fractions, and TLR5-mediated NF-κB luciferase activity was measured. The fraction numbers correspond to 3 minute fractions of reverse-phase FPLC eluted with a non-linear gradient of buffer B as shown. Fraction number "N" is control LB growth medium and "P" is the *L. monocytogenes* culture supernatant prior to chromatography. Fractions containing background activity (1), low activity (2) and high activity (3) as indicated in Fig. 3a were analyzed by SDS-PAGE and silver stain. Silver staining was performed according to established methods. Two bands with apparent molecular masses of 30-34 kDa were clearly enriched in the fraction containing the highest level of TLR5-stimulating activity (Fig. 3b, Lane 3). Proteins eluted from regions A, B,

and C of the SDS-PAGE gel, as indicated in Fig. 3b were assayed for TLR5-mediated NF-κB activation in CHO cells. In Fig. 3c, "Listeria" indicates *L. monocytogenes* culture supernatant. One of these bands, (Fig. 3b, band A), was trypsin-treated, subject to microcapillary HPLC-tandem mass spectrometry, and identified by comparison of peptide tandem mass spectra to sequences in a non redundant protein database using the computer program, SEQUEST27 (Fig. 4a). TLR5-stimulating activity was not recovered from any other section of the gel.

Thus, a TLR5-stimulating activity was purified from culture supernatants from *L. monocytogenes*.

EXAMPLE IV

Flagellin is a TLR5 Stimulus

This example shows that flagellin is a TLR5 stimulus purified from culture supernatants from *L. monocytogenes*.

As described above, a TLR5-stimulating activity was purified from *L. monocytogenes* culture supernatants using HPLC. The isolated polypeptide of band A in Figure 3b was trypsinized and identified by microcapillary HPLC-tandem mass spectrometry. Peaks corresponding to *L. monocytogenes* flagellin peptides are indicated in Figure 4a. Five sequences were identified (Fig. 4a) that correspond to flagellin, the product of the flaA gene of *L. monocytogenes* (Genbank Q02551). The location of these sequences within the protein is indicated in figure 4b.

Band B of Fig. 3b also is flagellin, which migrates as a doublet of approximately 30kDa on SDS-PAGE (Fig. 3b).

For analysis, bands A and B were excised from SDS-PAGE gels, dehydrated with acetonitrile, dried under reduced vacuum, and trypsin (12.5 ng/μL) was infused into the gel. The gel slice was allowed to incubate on ice for 45 min in the presence of trypsin and then excess trypsin removed and replaced with 50 mM ammonium bicarbonate and the gel slice incubated overnight at 37°C. Peptides were extracted by 3 washes with 5% acetic acid in 50% aqueous acetonitrile. The extractions were pooled and concentrated by vacuum centrifugation. The peptides were injected onto a C18 peptide trap cartridge (Michrom BioResources, Inc. Auburn, CA), desalted, and then injected onto a 75 μm (internal diameter) x 10 cm micro-capillary HPLC column (Magic C18; 5-μm packing; 100 A pore size; Michrom BioResources, Inc. Auburn, CA). The sample injection was made using a FAMOS autosampler (LCPackings, San Francisco, CA) coupled with an Agilent HP1100 Pump. Peptides were separated by a linear gradient of acetonitrile, and subjected to collision induced dissociation using an electrospray ionization-ion trap mass spectrometer (ESI-ITMS; ThermoQuest, San Jose, CA) in data-dependent mode with dynamic exclusion (Goodlett, et al. Anal. Chem. 72, 1112-1118 (2000)). Protein identification was accomplished by use of the SEQUEST computer program (Eng et al. J. Am. Soc. Mass. Spectrom. 5, 976-989 (1994)).

CHO cells expressing an NF-κB luciferase reporter and TLR5 or TLR2 were stimulated with 100 μl/ml

Listeria supernatant or 33 μg/ml purified *Salmonella* flagellin. Flagellin was purified from *Salmonella typhimurium* (TH4778 *fliB- fliC+*) by the procedure of Ibrahim et al., J. Clin. Microbiol. 22, 1040-1044 (1985). As shown in Figure 4c, flagellin stimulated TLR5-expressing CHO cells, but not TLR2-expressing CHO cells. The mean and standard deviation of quadruplicate samples are indicated. CHO cells were transfected as described in above Examples with the addition of 0.1 μg of pNeo/Tak, and stable populations of cells expressing the indicated TLR with the luciferase reporters were selected in 100 μg/ml G418. These cells were plated on 96-well plates at 100,000 cells/well, incubated overnight, and processed in luciferase assays as described above.

The observation that flagellin is the TLR5 ligand also is supported by the finding that the flagellated bacteria, *L. monocytogenes* and *P. aeruginosa*, stimulate TLR5, while the TOP10 strain of *E. coli*, that has lost its flagella, does not (Fig. 2b). Similarly, TLR5-stimulating activity was found in *B. subtilis, L. innocua, S. typhimurium* and *S. minnesota*, all flagellated bacteria, while non-flagellated bacteria such as *H. influenza*, did not activate TLR5.

Thus, the TLR5-stimulating activity purified from *L. monocytogenes culture* supernatants was identified as flagellin by tandem mass spectrometry.

EXAMPLE V

Flagellin Expression in Bacteria Reconstitutes TLR5-Stimulating Activity

This Example shows that flagellin expression in bacteria reconstitutes TLR-stimulating activity, and deletion of flagellin genes abrogates TLR5-stimulating activity.

To confirm that flagellin is the sole TLR5 ligand in bacteria, *E. coli* (TOP10) that secrete little TLR5 activity (Fig. 2b) were transformed with the cDNA of *L. monocytogenes* flagellin (*flaA*) under the control of an inducible promoter. TLR-expressing CHO cells were stimulated for 5 hours with *E.coli* culture supernatants (67 µl/ml) in which expression of *L. monocytogenes* flagellin was induced or repressed. In the control sample, CHO cells were stimulated with supernatants from induced *E.coli* containing the *L. monocytogenes* flagellin gene cloned in the reverse orientation. Supernatants of *E. coli* that were induced to express *L. monocytogenes flaA* contained substantial TLR5-stimulating activity (Fig. 5a), whereas supernatants from *E. coli* in which expression was repressed, or from *E. coli* expressing *flaA* in the reverse orientation, contained little TLR5 activity in CHO cells expressing an NF-KB luciferase reporter and TLR5 (Fig. 5a) or TLR2 (Fig 5b). CHO cells expressing an NF-κB luciferase reporter and TLR5 (c) or TLR2 (d) were stimulated for 5 hours with culture supernatants (100 µl/ml) from *S. typhimurium* lacking one copy of flagellin (*FliB- fliC+*) or both

copies of flagellin (*FliB+ FliC+*). Control is stimulation with LB medium. The mean and standard deviation of quadruplicate samples are indicated.

CHO cells were transfected with TLR2 and TLR5 expression plasmids as described above with the addition of 0.1 μg of pNeo/Tak, and stable populations of cells expressing the indicated TLR with the luciferase reporters were selected in 100 μg/ml G418. These cells were plated on 96-well plates at 100,000 cells/well, incubated overnight, and processed in luciferase assays as described above.

L. monocytogenes flagellin is not recognized by TLR2, since supernatants from *E. coli* expressing *flaA* did not show enhanced TLR2-dependent stimulation of CHO cells relative to supernatants from *E. coli* with repressed *flaA* expression (Fig. 5b). In addition to the experiments that demonstrate reconstitution of TLR5-stimulating activity by the expression of flagellin, a bacterium from which flagellin had been deleted was tested. It was observed that TLR5-stimulating activity was abrogated in the flagellin deleted strain. *S. typhimurium* possess two genes for flagellin, *fliB* and *fliC* (Fujita, J., J. Gen Microbiol. 76, 127-34 (1973)). Culture supernatants of *fliB- fliC+ S. typhimurium* contained TLR5-stimulating activity, while culture supernatants from *S. typhimurium* lacking both flagellins (*fliB- fliC-*) expressed no TLR5-stimulating activity (Fig. 5c). The lack of both flagellin genes had no effect on TLR2-stimulating activity (Fig. 5d). The observed TLR2-stimulating activity found in *S.*

typhimurium supernatants most likely was due to bacterial lipoproteins (Underhill, et al. Nature 401, 811-5 (1999); Brightbill et al., Science 285, 732-6 (1999)). These results indicate that flagellin is the sole TLR5-stimulating activity present in *S. typhimurium* culture supernatant.

Thus, TLR5-stimulating activity was elicited by introducing the flagellin gene into a non-flagellated bacterium, and abrogated by deleting the flagellin genes from a flagellated bacterium.

EXAMPLE VI

Flagellin-Induced System IL-6 Production in Mice

This example shows that TLR signaling is required for the *in vivo* immune response to flagellin.

To determine if TLR signaling is required for the *in vivo* immune response to flagellin, wild type mice and mice lacking a component of the TLR5 signal transduction pathway, MyD88, were injected with flagellin and systemic IL-6 production was monitored. MyD88 is an adaptor protein required for TLR5-mediated signal transduction (Aderem A. and Ulevitch, R.J., Nature 406:782-787, (2000); Brightbill, H.D. and Modlin. R.L., Immunology 101:1-10, (2000)).

$MyD88^{-/-}$ mice (129/SvJ x C57B1/6 background) were backcrossed for three generations with C57B1/6 mice (Adachi, O. et al. Immunity, 9:143-150 (1998)). Mice from the F_3 generation ($MyD88^{-/-}$, n=5) and littermate controls ($MyD88^{+/+}$, n=5) were injected i.p. with 30 μg

purified flagellin in 0.5 cc of saline. Blood was sampled at 0, 1, 2, 4 and 8 hours after injection, and IL-6 levels were determined by ELISA (Duoset, R&D Systems, Minneapolis, MN).

Figure 6 shows that flagellin induced systemic IL-6 within 2 h in wile type mice. By contrast, mice deficient in MyD88 were completely unresponsive to flagellin.

Therefore, flagellin stimulates TLR5-mediated responses *in vivo*.

Throughout this application various publications have been referenced. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention.

What is claimed is:

1. An immunomodulatory flagellin peptide comprising at least about 10 amino acids of substantially the amino acid sequence GAVQNRFNSAIT (SEQ ID NO:2), or a modification thereof, and having toll-like receptor 5 (TLR5) binding.

2. The flagellin peptide of claim 1, further comprising TLR5 stimulating activity.

3. The flagellin peptide of claim 1, further comprising an ADCC targeting molecule.

4. The flagellin peptide of claim 1, wherein said flagellin peptide comprises a peptide of *S. Typhimuriuml* flagellin.

5. A method of inducing an antigen-specific immune response in an individual comprising, administering to an individual an immunogenic amount of a vaccine, having an antigen and an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof.

6. The method of claim 5, wherein said antigen is selected from the group consisting of polypeptides, polysaccharides, pathologically aberrant cells and bacteria.

7. A method of inducing a TLR5-mediated response, comprising administering to a TLR5-containing cell an effective amount of an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof.

8. The method of claim 7, wherein said TLR5-mediated response is TLR5-induced modulation of cytokine amount or activity.

9. The method of claim 7, wherein said TLR5-mediated response is TLR5-induced increase in an amount of a cytokine selected from the group consisting of TNFα, IL-1 and IL-6.

10. The method of claim 7, wherein said TLR5-mediated response is TLR5-induced NF-κB activity.

11. A method of inducing an immune response in an individual having a pathological condition, comprising administering to said individual an immunogenic amount of an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof

12. The method of claim 11, wherein said pathological condition is selected from the group consisting of proliferative disease, autoimmune disease, infectious disease and inflammatory disease.

13. The method of claim 11, wherein said immunomodulatory flagellin peptide further comprises a heterologous amino acid sequence.

14. The method of claim 11, wherein said immunomodulatory flagellin peptide further comprises an ADCC targeting molecule.

15. A method of modulating an immune response in an individual having a pathological condition, comprising administering to said individual a combination of an immunogenic amount of an immunomodulatory flagellin peptide having at least about 10 amino acids of substantially the amino acid sequence of SEQ ID NO:2, or a modification thereof, and an immunomodulatory molecule.

16. The method of claim 15, wherein said immunomodulatory molecule is an antibody, cytokine or growth factor.

17. The method of claim 15, wherein said immunomodulatory flagellin peptide further comprises a heterologous amino acid sequence.

18. The method of claim 15, wherein said immunomodulatory flagellin peptide further comprises an ADCC targeting molecule.

19. The method of claim 15, wherein said pathological condition is selected from the group consisting of proliferative disease, autoimmune disease, infectious disease and inflammatory disease.

20. A method of inducing an immune response in a human individual having a pathological condition, comprising administering to said individual an immunogenic amount of a flagellin polypeptide, or modification thereof, wherein said flagellin polypeptide induces an immune response.

21. The flagellin polypeptide of claim 20, wherein said flagellin polypeptide comprises an *S. Typhimurium1* flagellin polypeptide.

22. The method of claim 20, wherein said pathological condition is selected from the group consisting of proliferative disease, autoimmune disease, infectious disease and inflammatory disease.

23. A screening composition comprising,
(a) a flagellin peptide of claim 1; and
(b) a TLR5 polypeptide or modification thereof, having a TLR5 activity.

24. The composition of claim 23, further comprising a detectably labeled flagellin peptide.

25. The composition of claim 23, wherein said TLR5 polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6, or a modification or fragment thereof having a TLR5 activity.

26. A method of screening for a TLR5 ligand, agonist or antagonist, comprising:

(a) contacting a TLR5 with a candidate compound in the presence of a flagellin polypeptide or immunomodulatory flagellin peptide under conditions wherein binding of said flagellin polypeptide or immunomodulatory flagellin peptide to said TLR5 produces a predetermined signal;

(b) determining the production of said predetermined signal in the presence of said candidate compound; and

(c) comparing said predetermined signal in the presence of said candidate compound with a predetermined signal in the absence of said candidate compound, wherein a difference between said predetermined signals in the presence and absence of said candidate compound indicates that said compound is a TLR5 ligand, agonist or antagonist.

27. The method of claim 26, wherein said predetermined signal is selected from the group consisting of polypeptide amount, polypeptide activity and transcriptional activity.

28. The method of claim 26, wherein said predetermined signal is amount of a cytokine selected from the group consisting of TNFα, IL-1 and IL-6.

29. The method of claim 27, wherein said transcriptional activity is NF-κB activity.

30. The method of claim 27, wherein said immunomodulatory flagellin peptide is a flagellin peptide of claim 1, or a modification thereof.

31. A bioassay for detecting bacterial contamination in a sample comprising,

(a) contacting said sample with a TLR5 under conditions wherein binding of a flagellin polypeptide or fragment thereof in said sample to said TLR5 produces a predetermined signal;

(b) determining the production of said predetermined signal in the presence and absence of said sample; and

(c) comparing said predetermined signal in the presence of said sample with a predetermined signal in the absence of said sample, wherein a difference between said predetermined signals in the presence and absence of said sample indicates that said sample contains flagellin.

32. The method of claim 31, wherein said sample is a product for animal consumption.

33. The method of claim 31, wherein said predetermined signal is selected from the group consisting of polypeptide amount, polypeptide activity and transcriptional activity.

34. The method of claim 31, wherein said predetermined signal is NF-κB activity.

35. The method of claim 31, wherein said predetermined signal is an amount a cytokine selected from the group consisting of TNFα, IL-1 and IL-6.

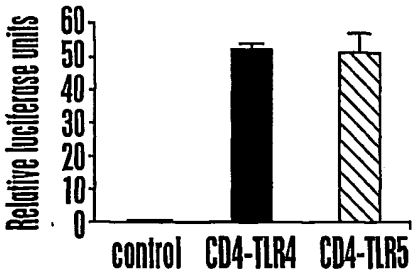


Figure 1A

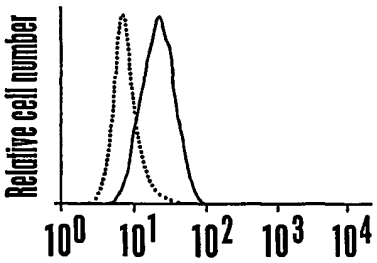


Figure 1B

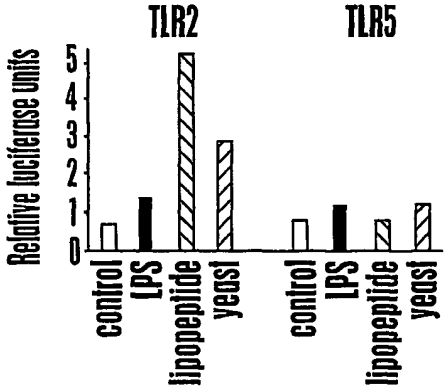


Figure 2A

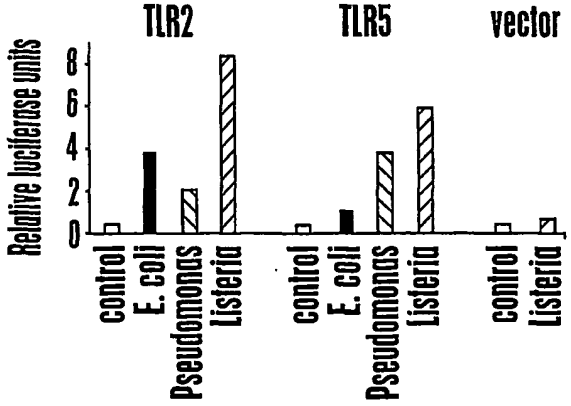
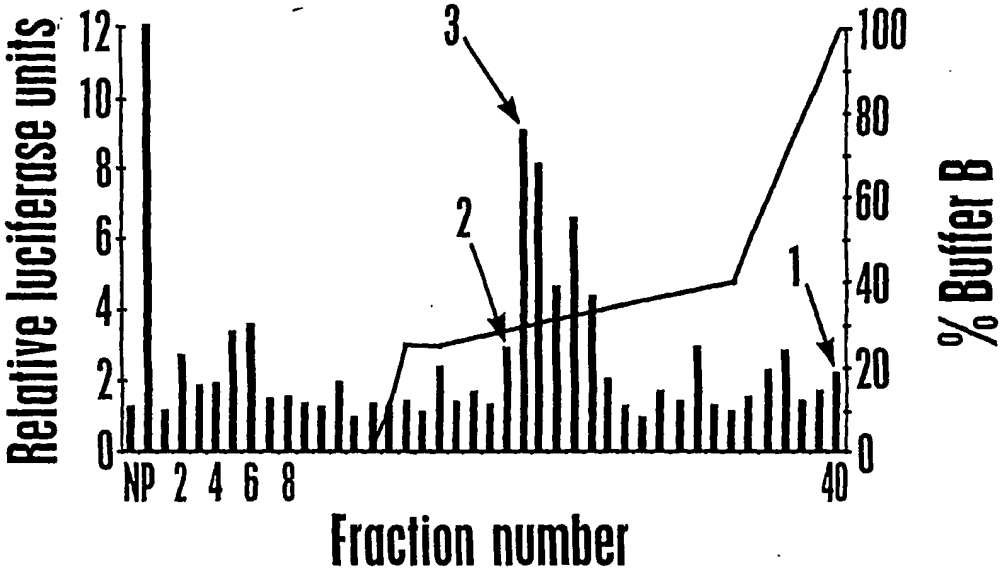


Figure 2B

Figure 3A



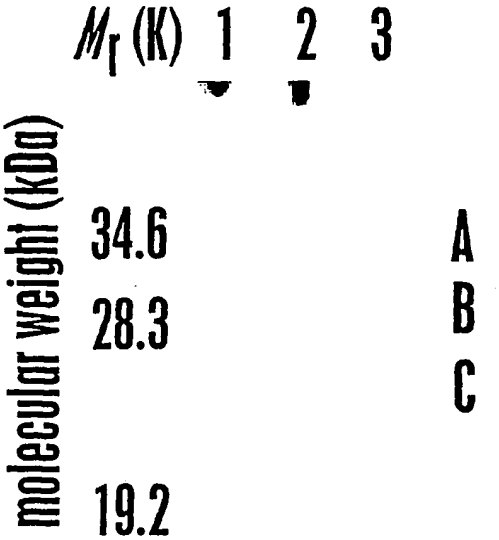


Figure 3B

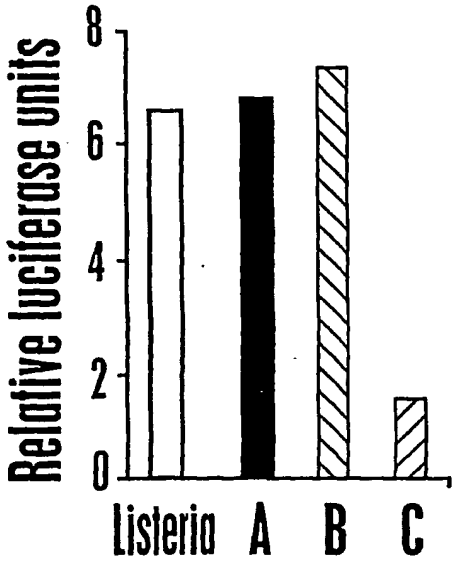


Figure 3C

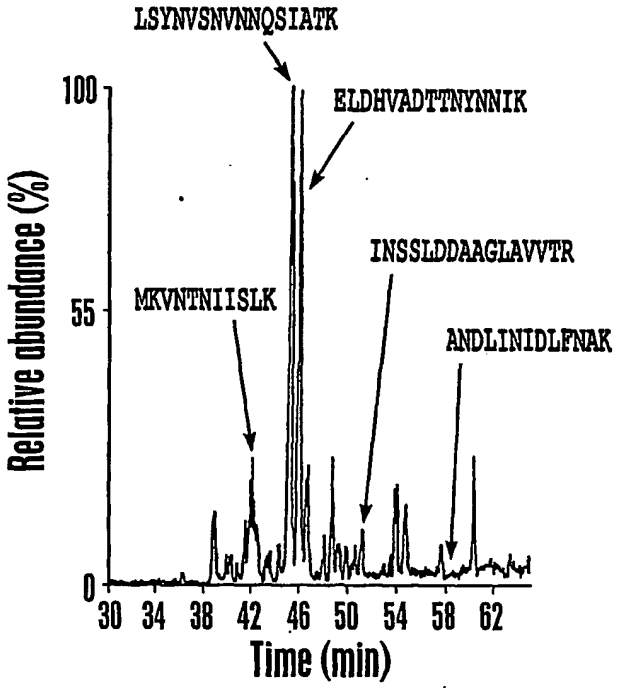


Fig. 4A

MKVNTNIISLKTQEYLRKNNEGMTQAQERLASGKRINSSLDD
AAGLAVVTRMNVKSTGLDAASKNSSMGIDLLQTADSALSSMS
SILQRMRQLAVQSSNGSFSDEDRKQYTAEFGSLIKELDHVAD
TTNYNNIKLLDQTATGAATQVSIQASDKANDLINIDLFNAKG
LSAGTITLGSGSTVAGYSALSVADADSSQEATEAIDELINNI
SNGRALLGAGMSRLSYNVSNVNNQSIATKASASSIEDADMAA
EMSEMTKYKILTQTSISMLSQANQTPQMLTQLINS

Fig. 4B

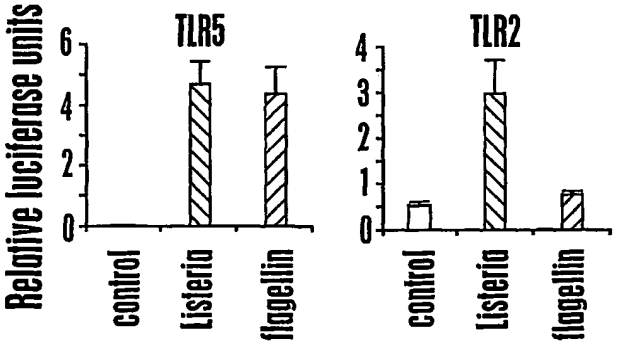


Fig. 4C

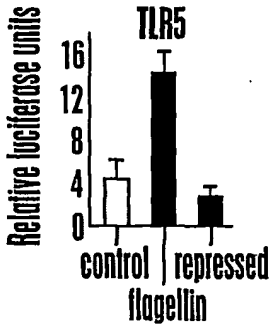


Fig. 5A

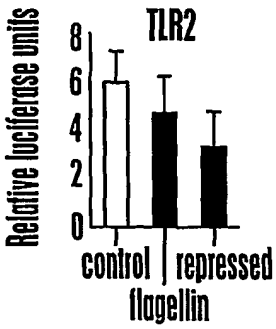


Fig. 5B

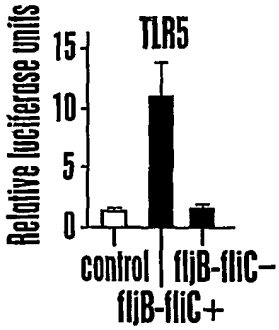


Fig. 5C

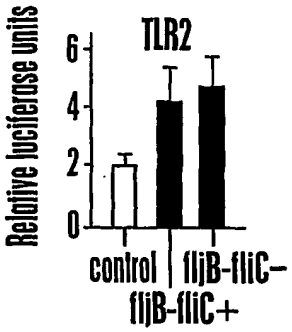


Fig. 5D

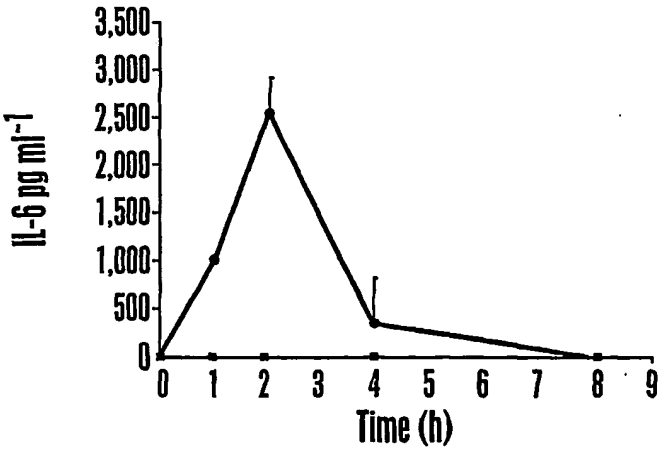
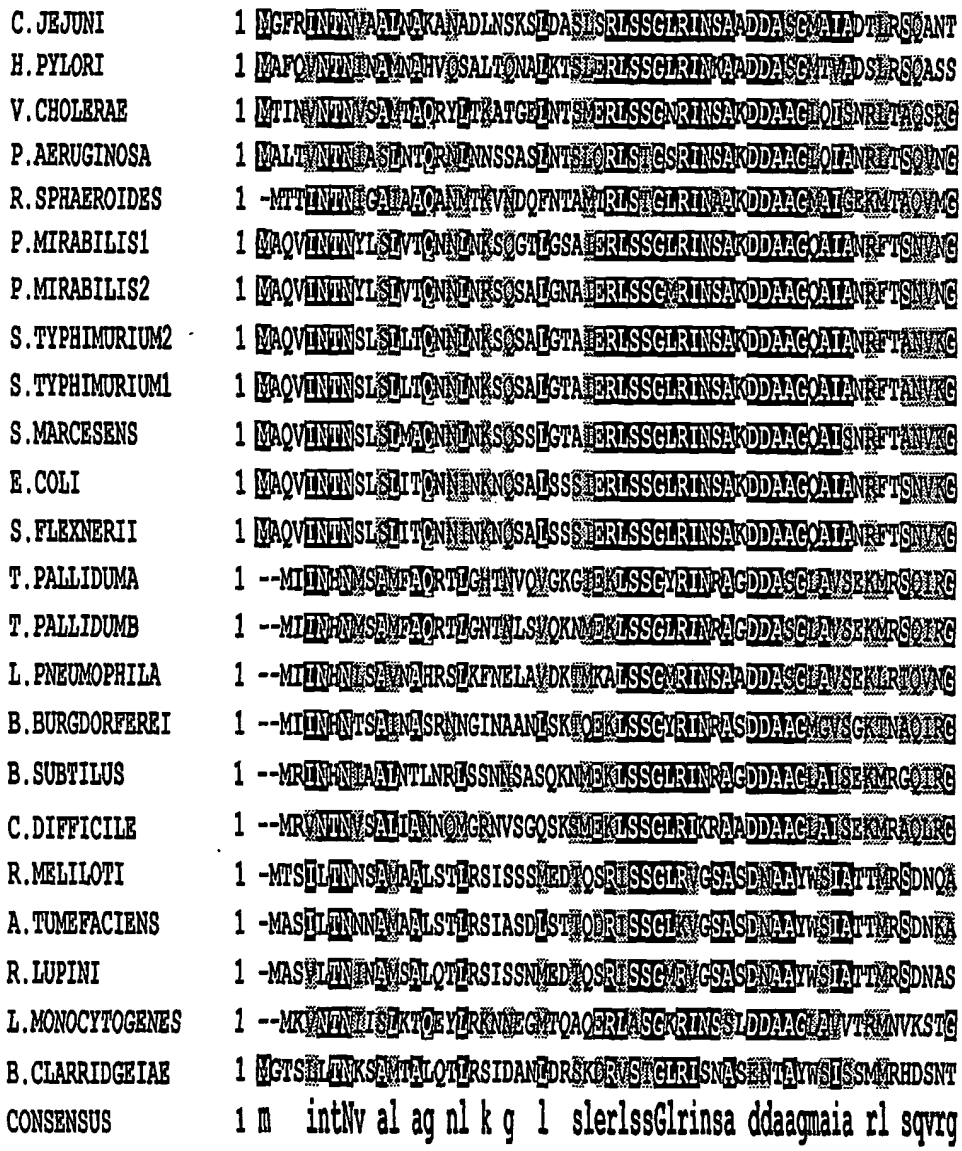


Fig. 6


C.JEJUNI 1 MGFRINTNVAALAQKANADLNSKSLDASLSRLSSGLRINSAADDASGMAIADTLRSQANT
H.PYLORI 1 MAFQVNTNIAAMNAHVQSALTQNALKTSLERLSSGLRINKAADDASGMTMADSLRSQASS
V.CHOLERAE 1 MTINVNTNVSAMTAQRYLTKATGELNTSMERLSSGNRINSAKDDAAGLQISNRLTAQSRG
P.AERUGINOSA 1 MALTVNTNIASLNTQRNLANNSSASLNTSLQRLSTGSRINSAKDDAAGLQIANRLTSQVNG
R.SPHAEROIDES 1 -MTTLNTNIGALAQANMTKVMDQFNTAMTRLSTGLRINAAKDDAAGMAISEKMTAQVMG
P.MIRABILIS1 1 MAQVINTNYLSLVTQNNLNKSQGTLGSAIERLSSGLRINSAKDDAAGQAIANRFTSNVNG
P.MIRABILIS2 1 MAQVINTNYLSLVTQNNLNKSQSALGNAIERLSSGYRINSAKDDAAGQAIANRFTSNVNG
S.TYPHIMURIUM2 1 MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANVKG
S.TYPHIMURIUM1 1 MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANVKG
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E.COLI 1 MAQVINTNSLSLITQNNLNKNQSALSSSIERLSSGLRINSAKDDAAGQAIANRFTSNVKG
S.FLEXNERII 1 MAQVINTNSLSLITQNNLNKNQSALSSSIERLSSGLRINSAKDDAAGQAIANRFTSNVKG
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T.PALLIDUMB 1 --MIINHNMSAMFAQRTLGNTNLSLQKNMEKLSSGLRINRAGDDASGLAVSEKMRSQIRG
L.PNEUMOPHILA 1 --MIINHNLSHVNAHRSLKFNELAVDKTMKALSSGYRINSAADDASGLAVSEKLRTQVNG
B.BURGDORFEREI 1 --MIINHNTSAINASRNNGINAANLSKTQEKLSSGYRINRASDDAAGMGVSGKTNAQIRG
B.SUBTILUS 1 --MRINHNIAALNTLNRLSSNMSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRG
C.DIFFICILE 1 --MRVNTNVSALIANQVGRNVSGQSKSMEKLSSGLRIKRAADDAAGLAISEKMRAQLRG
R.MELILOTI 1 -MTSLLTNNSAVAALSTLRSISSSMEDTQSRLSSGLRVGSASDNAAYWSIATTMRSDNQA
A.TUMEFACIENS 1 -MASLLTNNNAVAALSTLRSIASDLSTTQDRLSSGLKVGSASDNAAYWSIATTMRSDNKA
R.LUPINI 1 -MASVLTNNAAVSALQTLRSISSNMEDTQSRLSSGVRVGSASDNAAYWSIATTMRSDNAS
L.MONOCYTOGENES 1 --MKINTNIISLKTQEYLRKNMEGMTQAQERLASGKRINSSLDDAAGLAVTRKMNVKSTG
B.CLARRIDGEIAE 1 MGTSLLTNKSAMTALQTLRSIDANLDRSKGRVSTGLRISNASENTAYWSLSSMMRHDSNT
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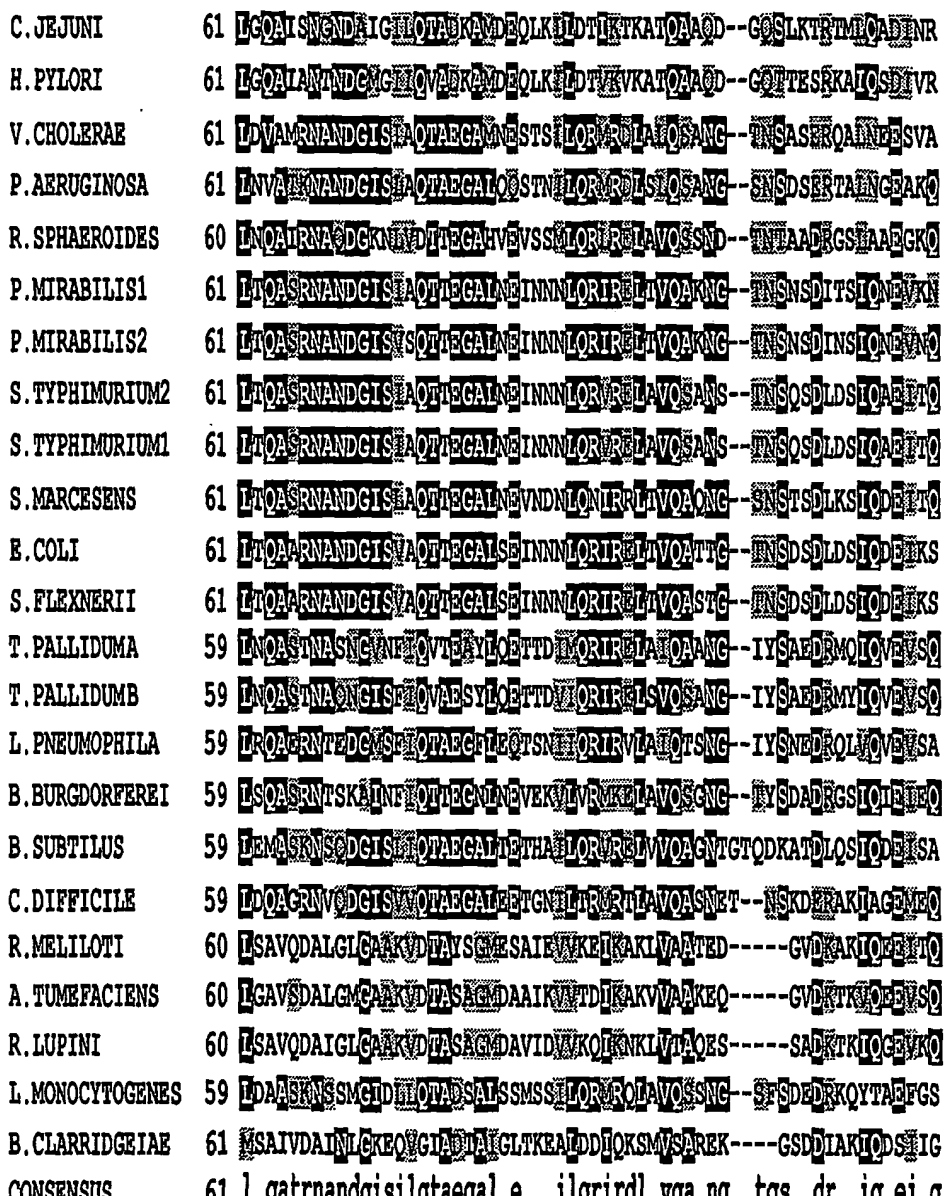
Figure 7AA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P.MIRABILIS2 61
S.TYPHIMURIUM2 61
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R.LUPINI 60
L.MONOCYTOGENES 59
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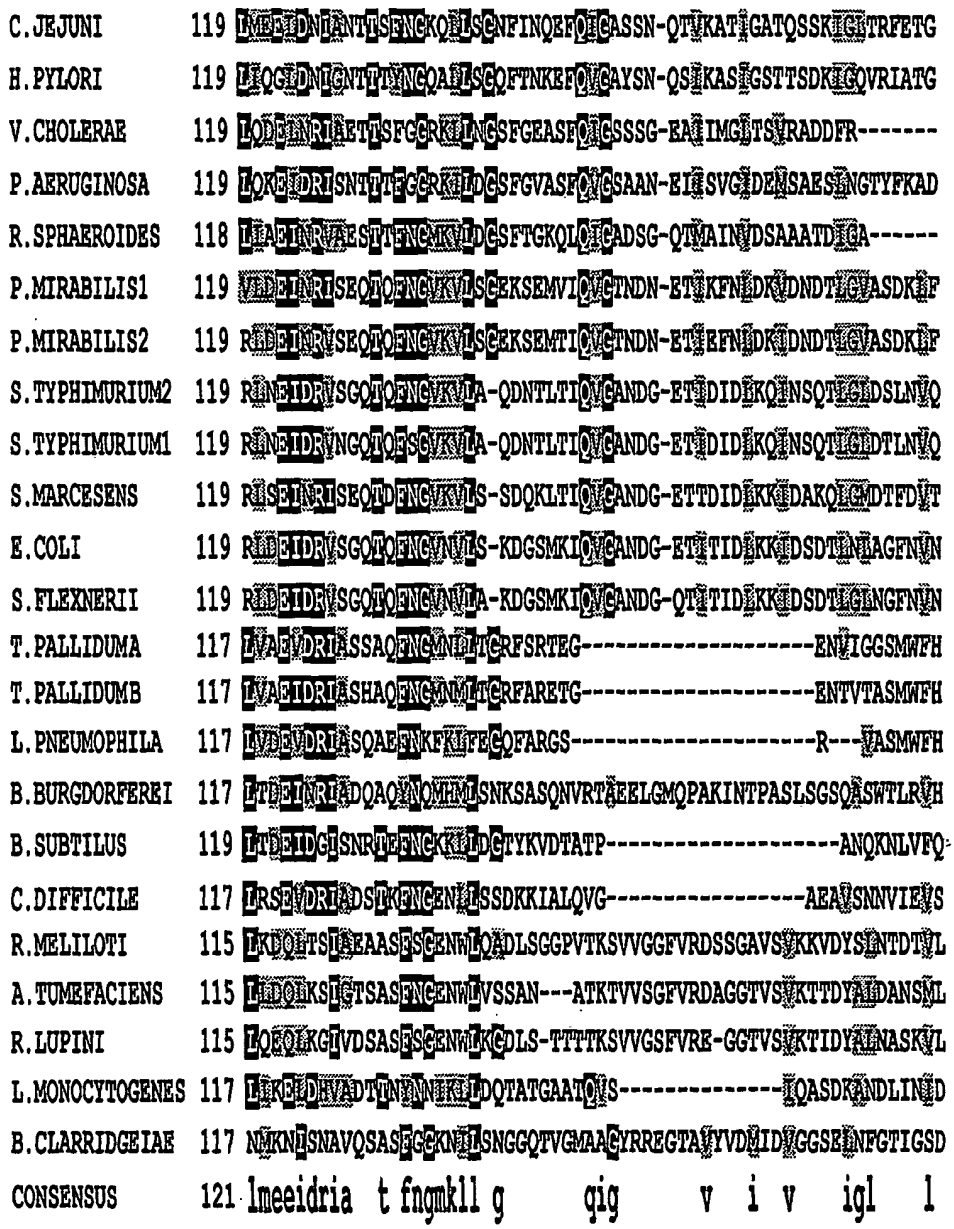
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V.CHOLERAE 119 LQDEINRIAETLSFGCRKILNGSFGEASFQICSSSG-EAIIMGITSVRADDFR-------
P.AERUGINOSA 119 LQKEIDRISNTVTFGCRKILDGSFGVASFQVCSAAN-EIISVGIDEMSAESINGTYFKAD
R.SPHAEROIDES 118 LIAEINRVAESTTFNCMKVLDGSFTGKQLQICADSG-QTMAINVDSAAATDIGA------
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P.MIRABILIS2 119 RLDEINRVSEQIQFNCVKVLSGEKSEMTIQVGTNDN-ETIEFNLDKQDNDTLGVASDKIF
S.TYPHIMURIUM2 119 RINEIDRVSGQIQFNCVKVLA-QDNTLTIQVGANDG-ETIDIDIKQINSQTLGIDSLNVQ
S.TYPHIMURIUM1 119 RINEIDRVNGQIQFSCVKVLA-QDNTLTIQVGANDG-ETIDIDIKQINSQTLGIDTLNVQ
S.MARCESENS 119 RISEINRISEQIDFNCVKVLS-SDQKLTIQIGANDG-ETTDIDIKKIDAKQLGMDTFDIT
E.COLI 119 RLDEIDRVSGQIQFNCVNVLS-KDGSMKIQVGANDG-ETITIDIKKIDSDTINIAGFNIN
S.FLEXNERII 119 RLDEIDRVSGQIQFNCVNVLA-KDGSMKIQVGANDG-QTITIDIKKIDSDTLGLNGFNIN
T.PALLIDUMA 117 LVAEVDRIASSAQFNCVNDLTCRFSRTEG----------ENVIGGSMWFH
T.PALLIDUMB 117 LVAEIDRIASHAQFNCMNMITCRFARETG----------ENTVTASMWFH
L.PNEUMOPHILA 117 LVDEVDRIASQAEFNKFKLTFGQFARGS----------R---VASMWFH
B.BURGDORFEREI 117 LTLELNRIADQAQINQMHMISNKSASQNVRTAEELGMQPAKINTPASLSGSQASWTLRVH
B.SUBTILUS 119 LTDEIDGISNRTEFNCKKLDGTYKVDTATP----------ANQKNLVFQ
C.DIFFICILE 117 LRSEVDRIADSTKFNGENLSSDKKIALQVG----------AEAVSNNVIEVS
R.MELILOTI 115 IKDQLTSLEAASFSGENWLQADLSGGPVTKSVVGGFVRDSSGAVSVKKVDYSLNTDTVL
A.TUMEFACIENS 115 LLDQLKSLGTSASFNGENWLVSSAN---ATKTVVSGFVRDAGGTVSVKTTDYALDANSML
R.LUPINI 115 LQEQLKGIVDSASFSGENWLKGDLS-TTTTKSVVGSFVRE-GGTVSIKTIDYALNASKVL
L.MONOCYTOGENES 117 LIKELDHVADTTNFNNIKLDQTATGAATQVS----------IQASDKANDLINID
B.CLARRIDGEIAE 117 NMKNLSNAVQSASFGGKNLLSNGGQTVGMAAGYRREGTAIYVDMIDVGGSEINFGTIGSD
CONSENSUS 121 lmeeidria t fngmkll g qig v i v igl l

Figure 7BA

```
C.JEJUNI          178 GRISTSGEVQFTLKNYNGIDDFQFOKVVISTSVGTGLGALADEINKNADKTG----VRAT
H.PYLORI          178 ALITASGDISLTFKQVDGVNDVTLESVKVSSSAGTGIGVLAEVTNKNSNRTG----VKAY
V.CHOLERAE        171 MGGQSFIAEQPKTKEWGVP-----------------------------------------
P.AERUGINOSA      178 GGGAVTAATASGTVDIAIG---------------------------------------
R.SPHAEROIDES         ---------------------------------------------------------
P.MIRABILIS1      178 DTKTEKKGVTAAG-------------------------------------------
P.MIRABILIS2      178 DAKTEKKGVTAAG-------------------------------------------
S.TYPHIMURIUM2    177 KAYDVKDTAVTTKAYANNGTTLDVSGLDDAAIKAATGGTNGTASVT-------GGAVKFD
S.TYPHIMURIUM1    177 QKYKVSDTAATVTGYADTTIALDNS-----TFKASATGLGGTDEKI-------DGDLKFD
S.MARCESENS       177 TKSAKAGAEIATG--------------------------------------------
E.COLI            177 GEGETANTAATLKDMVGLKLDNTGVTTAGVNRYIADKAVASSTDILNAVAGVDGSKVSTE
S.FLEXNERII       177 GGGAVANTAASKADLVAANATVVGNKYTVSAGYDAAKASDLLAGVS---D---GDTVQAT
T.PALLIDUMA       157 IGANMDQRMR------VY----------------------------------------
T.PALLIDUMB       157 IGANMDQRTR------AY----------------------------------------
L.PNEUMOPHILA     153 MGPNQNQRER------FY----------------------------------------
B.BURGDORFEREI    177 VGANQDEAIA------VN----------------------------------------
B.SUBTILUS        159 IGANATQQIS------VN----------------------------------------
C.DIFFICILE       160 LINTKGVLTT------RN----------------------------------------
R.MELILOTI        175 FDTTGN---TGILDKVYN----------------------------------------
A.TUMEFACIENS     172 YTEG-------------------------------------------------------
R.LUPINI          173 VDTRATGTKIGILDTAYTG-----------------------------------------
L.MONOCYTOGENES   163 LFNAKGLSAG-----------------------------------------------
B.CLARRIDGEIAE    177 GTTDMSQGVLGGIFGTSKG-----------------------------------------
CONSENSUS         181
```

Figure 7BB

```
C.JEJUNI          234 FTVETRGIAAVRAGATSDTFAINGVKIGKVDYKDGDANGALVAAINSVKDTTGVEASIDA
H.PYLORI          234 ASVITTSDVAVQSGSLGNLTLNGIHLGNIADIKKNDSDGRLVAAINAVTSETGVEAYTDQ
V.CHOLERAE        190 ----------------------------------------------------PTARDLKFEFTKK
P.AERUGINOSA      197 ---------------------------------------------------TTGGSAVNVKVDM
R.SPHAEROIDES         ------------------------------------------------------------
P.MIRABILIS1      191 --------------------------AG--------------------VTDAKKINA
P.MIRABILIS2      191 --------------------------DA--------------------IDANALGIS
S.TYPHIMURIUM2    230 ADNNKYFVTIGGFTGADAAKNG--DYEVNVATDGTVTLAAGATKTTMPAGATTKTEVQEL
S.TYPHIMURIUM1    225 DTTGKYYAKVTVTG--GTGKDG--YYEVSVDKTNGEVTLAAVTPATVTTATALSGKMYSA
S.MARCESENS       190 --------------------------T--------------------KITVDS--DA
E.COLI            237 ADVGFGAAAPGTPVEYTYHKDTNTYTASASVDATQLAAFLNPEAGGTTAATVSIGNGTTA
S.FLEXNERII       231 INNGFGTAASATNYKYDSASKS-YSFDTTTASAADVQKYLTPGVGDTAKGTTTIDG---S
T.PALLIDUMA       169 ----------------------------------------------------IGTMTAVA
T.PALLIDUMB       169 ----------------------------------------------------IGTMTAAA
L.PNEUMOPHILA     165 ----------------------------------------------------IGTMTSKA
B.BURGDORFEREI    189 ----------------------------------------------------IYAANVAN
B.SUBTILUS        171 ----------------------------------------------------IEDMGADA
C.DIFFICILE       172 ----------------------------------------------------VNSANIDA
R.MELILOTI        190 ----------------------------------------------VSQASVTLPVNV
A.TUMEFACIENS     176 ------------------------------------------------------T--
R.LUPINI          192----------------------------------------------LNANTVTVDINK
L.MONOCYTOGENES   173 ------------------------------------------------------------
B.CLARRIDGEIAE    196 ----------------------------------------------DEGEDVVGKGIGA
CONSENSUS         241
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Figure 7CA

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C.JEJUNI          294 NGQLLLTSREGRGIKIDGNIGGGAFINADMKENYGRLSLVKNDGKDILISGSNLSSAGFG
H.PYLORI          294 KGRLNLRSIDGRGIEIK----------TDSVSNGPSALTMVNGGQDLTKGSTNYGRLSLT
V.CHOLERAE        203 DG----EAVVLDIIAKDGD----------------DIEELA---------TYINGQTD
P.AERUGINOSA      210 KGNETAEQAAAKIAAAVND----------------ANVGIG---------AFSDGDTI
R.SPHAEROIDES         ------------------------------------------------------------
P.MIRABILIS1      202 AATLDMMVSLVKEFNLDG----------------KPVTDK--------FIVTKGGKD
P.MIRABILIS2      202 GSKKYVTGISVKEYKVDG----------------KVSSDK--------VVLNDGSDD
S.TYPHIMURIUM2    288 KDTPAVVSADAKNALIAGGV------DATDANGAELVKMSYTDKNGKTIEGGYALKAGDK
S.TYPHIMURIUM1    281 NPDSDIAKAALTAAGVTG----------TASVVKMSYTDNNGKTIDGGLAVKVGDD
S.MARCESENS       199 T-----KQADADVTGLAKG----------QTLVSG----------TDADGKSA
E.COLI            297 QEQKVIIAKDGSLTAADDG----------AALYLDDTGNLSKTN-AGTDTQAKLS
S.FLEXNERII       287 -AQDVQISSDGKITASNG----------DKLYIDTTGRLTKNGSGASLTEASLS
T.PALLIDUMA       177 LG------------------------------------------------------
T.PALLIDUMB       177 LG------------------------------------------------------
L.PNEUMOPHILA     173 LK------------------------------------------------------
B.BURGDORFEREI    197 LFSGEGAQAAQTAPVQEGA-------------------------------------
B.SUBTILUS        179 LGIKEADG----------------------------------------------
C.DIFFICILE       180 MS------------------------------------------------------
R.MELILOTI        202 NGTTSEYTVGAYNVDDLID----------ASATFDGDYANVGAGALAGDYVKVQG
A.TUMEFACIENS     177 ------------------------------------------------------
R.LUPINI          204 GGVITQASVRAYSTDEMLS----------LGAKVDGANSNVAVGGGSAFVKVDGS
L.MONOCYTOGENES   173 ------------------------------------------------------
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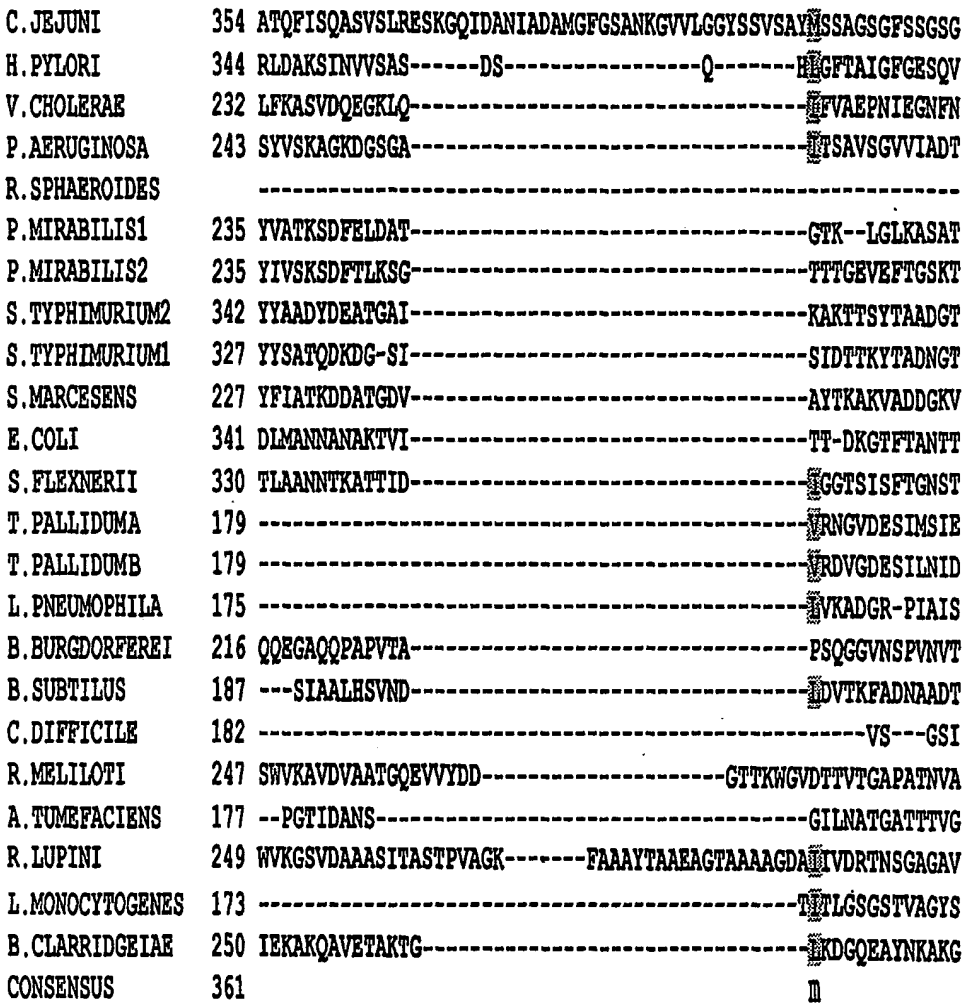
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R.SPHAEROIDES
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P.MIRABILIS2 235 YIVSKSDFTLKSG----------TTTGEVEFTGSKT
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S.MARCESENS 227 YFIATKDDATGDV----------AYTKAKVADDGKV
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S.FLEXNERII 330 TLAANNTKATTID----------GGTSISFTGNST
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T.PALLIDUMB 179 ----------RDVGDESILNID
L.PNEUMOPHILA 175 ----------VKADGR-PIAIS
B.BURGDORFEREI 216 QQEGAQQPAPVTA----------PSQGGVNSPVNVT
B.SUBTILUS 187 ---SIAALHSVND----------DVTKFADNAADT
C.DIFFICILE 182 ----------VS---GSI
R.MELILOTI 247 SWVKAVDVAATGQEVVYDD----------GTTKWGVDTTVTGAPATNVA
A.TUMEFACIENS 177 --PGTIDANS----------GILNATGATTTVG
R.LUPINI 249 WVKGSVDAAASITASTPVAGK-------FAAAYTAAEAGTAAAAGDAIVDRTNSGAGAV
L.MONOCYTOGENES 173 ----------TTLGSGSTVAGYS
B.CLARRIDGEIAE 250 IEKAKQAVETAKTG----------KDGQEAYNKAKG
CONSENSUS 361

Figure 7DA

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P.AERUGINOSA      269 GSTGVGTAAGVAPSA----------------------------------------------
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S.TYPHIMURIUM1    352 SKTALNKLGGADGKTEVVTIDGKTYNAS--------------------------------
S.MARCESENS       253 TDSGTDAG-----------------------------------------------------
E.COLI            366 KFDGVDISVDASTFANAVKNETYTATVG--VTLPATYTVNNGTAASAYLVDGKVSKTP--
S.FLEXNERII       356 TPDTITYSVTGAKVDQAAFDKAVSTSGNNVDFTTAGYSVNGTTGAVTKGVDSVYVDNNEA
T.PALLIDUMA       192 --TADSAN-------------------------------------------------------
T.PALLIDUMB       192 --DPEKAN-------------------------------------------------------
L.PNEUMOPHILA     187 --SPGEAN-------------------------------------------------------
B.BURGDORFEREI    242 --TTVDAN-------------------------------------------------------
B.SUBTILUS        210 --ADIGFD-------------------------------------------------------
C.DIFFICILE       187 --GTEAAS-------------------------------------------------------
R.MELILOTI        286 APASIATIDITIAAQ-----------------------------------------------
A.TUMEFACIENS     198 AKTYTQISVLDMNVG----------------------------------------------
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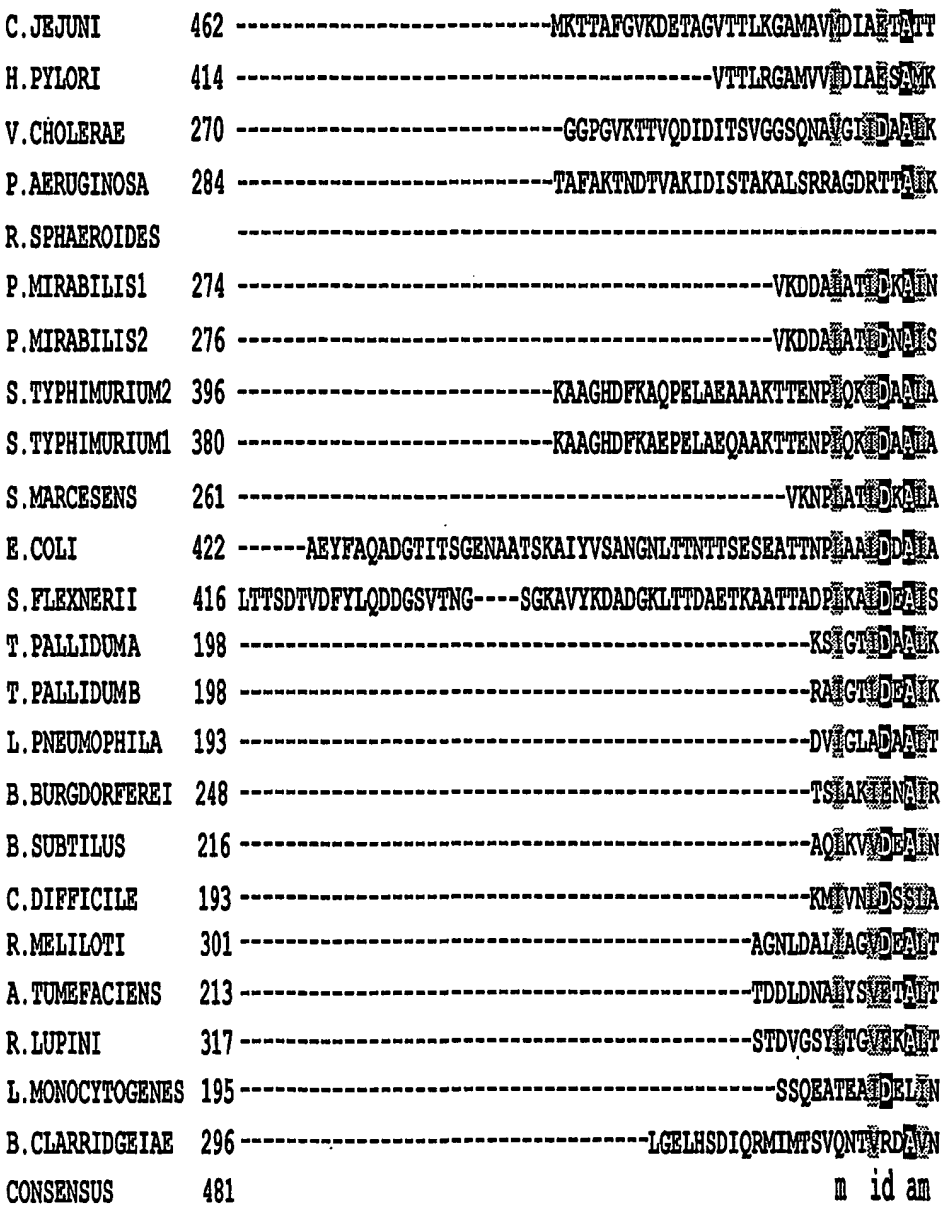
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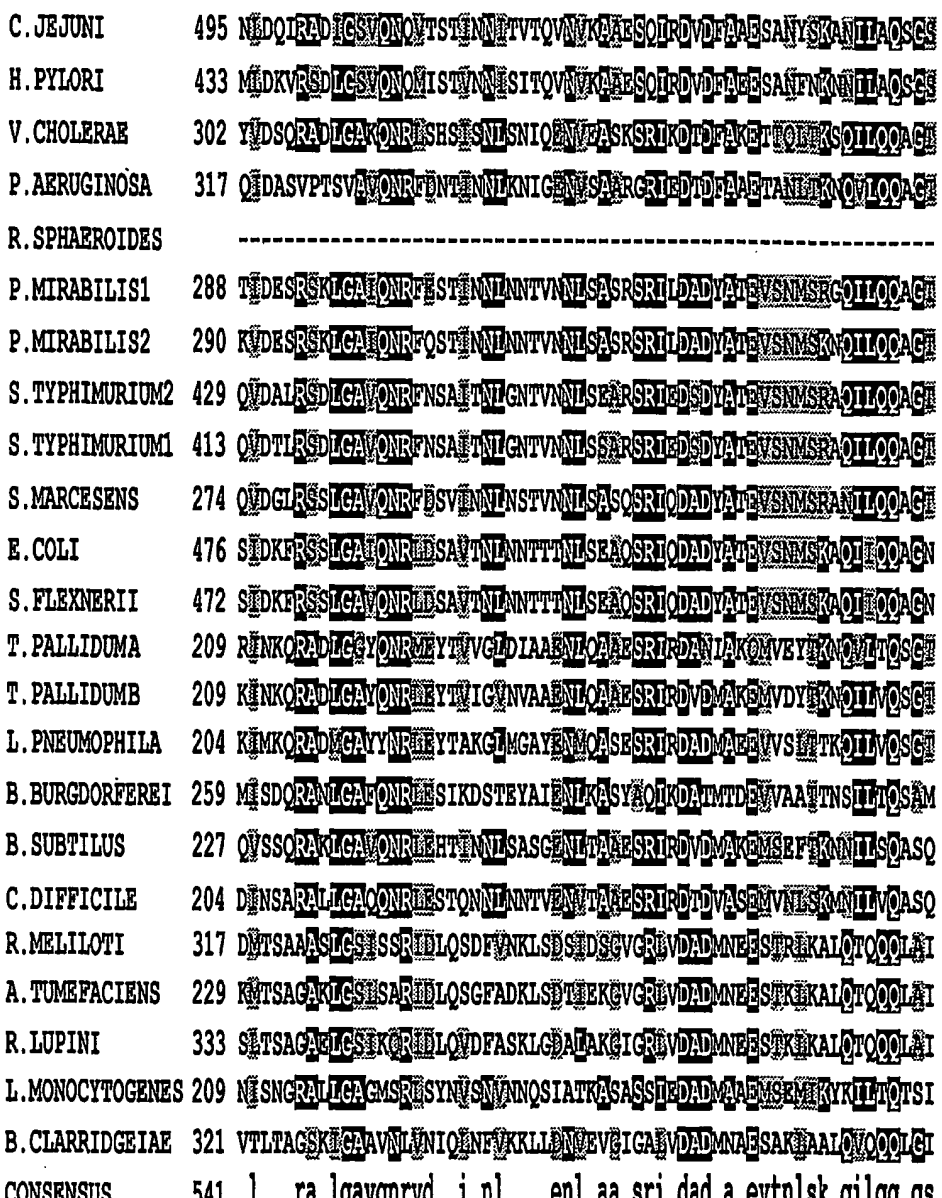
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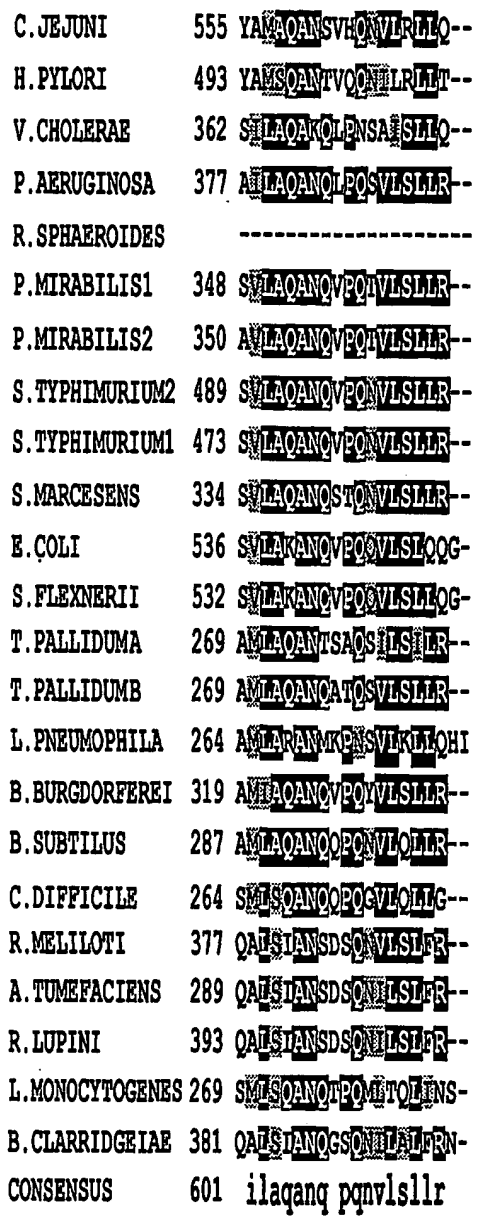
Figure 7EB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L.MONOCYTOGENES 269 SMLSQANQIPQMITQLINS-
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ggtccaaggg acagtaccct ccagtgctgg ggacccatgt gccgagcctc aggctgtgat 960
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                                        Met Ala Cys Gln Leu Asp
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ttg ctc ata ggt gtg atc ttc atg gcc agc ccc gtg ttg gta ata tct   1064
Leu Leu Ile Gly Val Ile Phe Met Ala Ser Pro Val Leu Val Ile Ser
            10                  15                  20

ccc tgt tct tca gac ggc agg ata gcc ttt ttc cga ggc tgt aac ctc   1112
Pro Cys Ser Ser Asp Gly Arg Ile Ala Phe Phe Arg Gly Cys Asn Leu
        25                  30                  35

acc cag att ccc tgg atc ctc aat act acc act gag agg ctc ctg ctc   1160
Thr Gln Ile Pro Trp Ile Leu Asn Thr Thr Thr Glu Arg Leu Leu Leu
    40                  45                  50

agc ttc aac tat atc agt atg gtg gtt gcc aca tca ttt cca ctc ctg   1208
Ser Phe Asn Tyr Ile Ser Met Val Val Ala Thr Ser Phe Pro Leu Leu
55                  60                  65                  70

gag cgg ctc cag ttg ctg gag ctg ggg acc cag tat gct aac ttg acc   1256
Glu Arg Leu Gln Leu Leu Glu Leu Gly Thr Gln Tyr Ala Asn Leu Thr
                75                  80                  85

att ggt cca ggg gct ttc aga aac ctg ccc aat ctt agg atc ttg gac   1304
Ile Gly Pro Gly Ala Phe Arg Asn Leu Pro Asn Leu Arg Ile Leu Asp
            90                  95                  100

ttg ggc caa agc cag atc gaa gtc ttg aat cga gat gcc ttt caa ggt   1352
Leu Gly Gln Ser Gln Ile Glu Val Leu Asn Arg Asp Ala Phe Gln Gly
        105                 110                 115

ctg ccc cat ctc ttg gaa ctt cgg ctg ttt tcc tgt gga ctc tcc agt   1400
Leu Pro His Leu Leu Glu Leu Arg Leu Phe Ser Cys Gly Leu Ser Ser
    120                 125                 130

gct gtg tta agt gac ggt tac ttc aga aat cta tat tca tta gct cgc   1448
Ala Val Leu Ser Asp Gly Tyr Phe Arg Asn Leu Tyr Ser Leu Ala Arg
135                 140                 145                 150
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tta gac cta tct ggc aac cag att cac agc ctc cgc ctc cat tct tca   1496
Leu Asp Leu Ser Gly Asn Gln Ile His Ser Leu Arg Leu His Ser Ser
                155                 160                 165

ttc cgg gaa ctg aat tcc tta agc gac gta aat ttt gct ttc aac caa   1544
Phe Arg Glu Leu Asn Ser Leu Ser Asp Val Asn Phe Ala Phe Asn Gln
            170                 175                 180

ata ttc act ata tgt gaa gat gaa ctc gag cct ctg cag ggc aaa aca   1592
Ile Phe Thr Ile Cys Glu Asp Glu Leu Glu Pro Leu Gln Gly Lys Thr
        185                 190                 195

ctg tct ttc ttt ggc ctc aaa tta act aag ctg ttc agc aga gtc tct   1640
Leu Ser Phe Phe Gly Leu Lys Leu Thr Lys Leu Phe Ser Arg Val Ser
    200                 205                 210

gtg ggc tgg gag aca tgc agg aac ccc ttc aga ggc gtg agg cta gaa   1688
Val Gly Trp Glu Thr Cys Arg Asn Pro Phe Arg Gly Val Arg Leu Glu
215                 220                 225                 230

act cta gat ctt tct gaa aat ggc tgg acg gtg gac atc aca agg aac   1736
Thr Leu Asp Leu Ser Glu Asn Gly Trp Thr Val Asp Ile Thr Arg Asn
                235                 240                 245

ttc agc aac atc atc cag gga agc cag att tcc tct ttg att ctt aaa   1784
Phe Ser Asn Ile Ile Gln Gly Ser Gln Ile Ser Ser Leu Ile Leu Lys
            250                 255                 260

cac cac atc atg ggt cct ggc ttt ggc ttc cag aac atc aga gat cct   1832
His His Ile Met Gly Pro Gly Phe Gly Phe Gln Asn Ile Arg Asp Pro
        265                 270                 275

gac cag agc aca ttt gcc agc ctg gcc aga agt tcg gtg ctg caa ctg   1880
Asp Gln Ser Thr Phe Ala Ser Leu Ala Arg Ser Ser Val Leu Gln Leu
    280                 285                 290

gac ctt tcg cac ggc ttt atc ttc tcc ttg aat cct cga ctg ttt ggg   1928
Asp Leu Ser His Gly Phe Ile Phe Ser Leu Asn Pro Arg Leu Phe Gly
295                 300                 305                 310

aca ctg aag gat ttg aag atg ctg aac ctt gcc ttc aac aag ata aac   1976
Thr Leu Lys Asp Leu Lys Met Leu Asn Leu Ala Phe Asn Lys Ile Asn
                315                 320                 325

aag att gga gag aat gcc ttt tat ggg ctt gac agc ctc cag gtt ctc   2024
Lys Ile Gly Glu Asn Ala Phe Tyr Gly Leu Asp Ser Leu Gln Val Leu
            330                 335                 340

aat cta tcc tat aat ctt ttg ggg gaa ctc tat aat tcc aac ttc tat   2072
Asn Leu Ser Tyr Asn Leu Leu Gly Glu Leu Tyr Asn Ser Asn Phe Tyr
        345                 350                 355

ggg ctt cct aga gta gcc tac gtt gac ctt caa agg aac cac att ggg   2120
Gly Leu Pro Arg Val Ala Tyr Val Asp Leu Gln Arg Asn His Ile Gly
    360                 365                 370
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atc att caa gac caa aca ttc aga tta tta aaa acg tta caa acc tta   2168
Ile Ile Gln Asp Gln Thr Phe Arg Leu Leu Lys Thr Leu Gln Thr Leu
375                 380                 385                 390

gat ctc cgt gac aat gct ctt aag gcc att ggt ttt att cca agc ata   2216
Asp Leu Arg Asp Asn Ala Leu Lys Ala Ile Gly Phe Ile Pro Ser Ile
                395                 400                 405

cag atg gtc ctc ctg gga ggc aat aag ctg gtc cat ttg cca cac atc   2264
Gln Met Val Leu Leu Gly Gly Asn Lys Leu Val His Leu Pro His Ile
            410                 415                 420

cac ttt act gcc aac ttc cta gag tta tct gaa aac agg cta gaa aac   2312
His Phe Thr Ala Asn Phe Leu Glu Leu Ser Glu Asn Arg Leu Glu Asn
        425                 430                 435

ctg tcc gac ctc tac ttc ctc ctg cga gtc ccc cag ctc cag ttt ctc   2360
Leu Ser Asp Leu Tyr Phe Leu Leu Arg Val Pro Gln Leu Gln Phe Leu
    440                 445                 450

atc ttg aat cag aat cgc ctt tcg tca tgc aag gca gcc cac act ccc   2408
Ile Leu Asn Gln Asn Arg Leu Ser Ser Cys Lys Ala Ala His Thr Pro
455                 460                 465                 470

tcg gag aac cca agc tta gaa cag ctt ttc ctt aca gag aat atg ctg   2456
Ser Glu Asn Pro Ser Leu Glu Gln Leu Phe Leu Thr Glu Asn Met Leu
                475                 480                 485

cag ctg gcc tgg gag acc ggc ctc tgt tgg gat gtt ttt caa ggc ctt   2504
Gln Leu Ala Trp Glu Thr Gly Leu Cys Trp Asp Val Phe Gln Gly Leu
            490                 495                 500

tcc cgc ctc cag att ctt tac ctg agt aat aac tac ctt aat ttc ctt   2552
Ser Arg Leu Gln Ile Leu Tyr Leu Ser Asn Asn Tyr Leu Asn Phe Leu
        505                 510                 515

cca cct ggg ata ttt aac gac ctg gtt gca tta cgg atg ctt agt ctt   2600
Pro Pro Gly Ile Phe Asn Asp Leu Val Ala Leu Arg Met Leu Ser Leu
    520                 525                 530

agt gct aac aag ctg acc gtg ctc tct ccg ggc agt tta cct gct aat   2648
Ser Ala Asn Lys Leu Thr Val Leu Ser Pro Gly Ser Leu Pro Ala Asn
535                 540                 545                 550

tta gag att ctc gac ata tct aga aat cag ctt ttg tgt cct gac cct   2696
Leu Glu Ile Leu Asp Ile Ser Arg Asn Gln Leu Leu Cys Pro Asp Pro
                555                 560                 565

gct ttg ttt tct tcg ctt cgt gtt ttg gac ata act cat aac gag ttc   2744
Ala Leu Phe Ser Ser Leu Arg Val Leu Asp Ile Thr His Asn Glu Phe
            570                 575                 580

gtc tgc aac tgt gaa ctt agc act ttt atc tcc tgg ctc aac caa acc   2792
Val Cys Asn Cys Glu Leu Ser Thr Phe Ile Ser Trp Leu Asn Gln Thr
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        585                 590                 595

aac gtc acc ctg ttc ggc tct cct gca gac gtg tat tgc atg tac cct   2840
Asn Val Thr Leu Phe Gly Ser Pro Ala Asp Val Tyr Cys Met Tyr Pro
    600                 605                 610

aac tca ctg cta ggg ggc tcc ctc tac aac ata tcc acc gaa gac tgc   2888
Asn Ser Leu Leu Gly Gly Ser Leu Tyr Asn Ile Ser Thr Glu Asp Cys
615                 620                 625                 630

gat gaa gag gaa gcc atg cgg tcc cta aag ttt tcc ctt ttc atc ctg   2936
Asp Glu Glu Glu Ala Met Arg Ser Leu Lys Phe Ser Leu Phe Ile Leu
                635                 640                 645

tgc acg gtc act ttg act cta ttc ctc gtc atc acc ctt gta gtc ata   2984
Cys Thr Val Thr Leu Thr Leu Phe Leu Val Ile Thr Leu Val Val Ile
            650                 655                 660

aag ttc cgg gga atc tgt ttc ctg tgc tat aag acc atc cag aag ctg   3032
Lys Phe Arg Gly Ile Cys Phe Leu Cys Tyr Lys Thr Ile Gln Lys Leu
        665                 670                 675

gtg ttc aag gac aag gtc tgg agt ttg gaa cct ggt gca tat aga tat   3080
Val Phe Lys Asp Lys Val Trp Ser Leu Glu Pro Gly Ala Tyr Arg Tyr
    680                 685                 690

gat gcc tac ttc tgc ttc agc agc aaa gac ttt gaa tgg gca cag aat   3128
Asp Ala Tyr Phe Cys Phe Ser Ser Lys Asp Phe Glu Trp Ala Gln Asn
695                 700                 705                 710

gct ttg ctc aaa cac ctg gat gct cac tac agt tcc cga aac agg ctc   3176
Ala Leu Leu Lys His Leu Asp Ala His Tyr Ser Ser Arg Asn Arg Leu
                715                 720                 725

agg cta tgc ttt gaa gaa aga gac ttc att ccg ggg gaa aac cat atc   3224
Arg Leu Cys Phe Glu Glu Arg Asp Phe Ile Pro Gly Glu Asn His Ile
            730                 735                 740

tcc aac atc cag gcg gct gtc tgg ggc agc agg aag acg gtg tgt cta   3272
Ser Asn Ile Gln Ala Ala Val Trp Gly Ser Arg Lys Thr Val Cys Leu
        745                 750                 755

gtg agc aga cac ttc ctg aag gat ggt tgg tgc ctg gag gcc ttc agg   3320
Val Ser Arg His Phe Leu Lys Asp Gly Trp Cys Leu Glu Ala Phe Arg
    760                 765                 770

tat gcc cag agc cgg agt ctg tct gac ctc aag agc att ctc atc gtg   3368
Tyr Ala Gln Ser Arg Ser Leu Ser Asp Leu Lys Ser Ile Leu Ile Val
775                 780                 785                 790

gtg gtg gtg gga tcg ctg tcc cag tat cag ctg atg aga cat gag acc   3416
Val Val Val Gly Ser Leu Ser Gln Tyr Gln Leu Met Arg His Glu Thr
                795                 800                 805

atc aga ggg ttt ctg caa aag caa cag tac ttg agg tgg cct gaa gac   3464
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Ile Arg Gly Phe Leu Gln Lys Gln Gln Tyr Leu Arg Trp Pro Glu Asp
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ctc cag gat gtt ggc tgg ttt ctc gat aaa ctc tcc gga tgc att cta   3512
Leu Gln Asp Val Gly Trp Phe Leu Asp Lys Leu Ser Gly Cys Ile Leu
        825                 830                 835

aag gaa gaa aaa gga aag aaa aga agc agt tcc atc cag ttg cga acc   3560
Lys Glu Glu Lys Gly Lys Lys Arg Ser Ser Ser Ile Gln Leu Arg Thr
    840                 845                 850

ata gca acc att tcc tagcaggagc gcctcctagc agaagtgcaa gcatcgtaga   3615
Ile Ala Thr Ile Ser
855

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Phe Arg Gly Cys Asn Leu Thr Gln Ile Pro Trp Ile Leu Asn Thr Thr
        35                  40                  45
Thr Glu Arg Leu Leu Leu Ser Phe Asn Tyr Ile Ser Met Val Val Ala
    50                  55                  60
Thr Ser Phe Pro Leu Leu Glu Arg Leu Gln Leu Leu Glu Leu Gly Thr
65                  70                  75                  80
Gln Tyr Ala Asn Leu Thr Ile Gly Pro Gly Ala Phe Arg Asn Leu Pro
                85                  90                  95
Asn Leu Arg Ile Leu Asp Leu Gly Gln Ser Gln Ile Glu Val Leu Asn
            100                 105                 110
Arg Asp Ala Phe Gln Gly Leu Pro His Leu Leu Glu Leu Arg Leu Phe
        115                 120                 125
Ser Cys Gly Leu Ser Ser Ala Val Leu Ser Asp Gly Tyr Phe Arg Asn
    130                 135                 140
Leu Tyr Ser Leu Ala Arg Leu Asp Leu Ser Gly Asn Gln Ile His Ser
145                 150                 155                 160
Leu Arg Leu His Ser Ser Phe Arg Glu Leu Asn Ser Leu Ser Asp Val
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                165                 170                 175
Asn Phe Ala Phe Asn Gln Ile Phe Thr Ile Cys Glu Asp Glu Leu Glu
            180                 185                 190
Pro Leu Gln Gly Lys Thr Leu Ser Phe Phe Gly Leu Lys Leu Thr Lys
        195                 200                 205
Leu Phe Ser Arg Val Ser Val Gly Trp Glu Thr Cys Arg Asn Pro Phe
    210                 215                 220
Arg Gly Val Arg Leu Glu Thr Leu Asp Leu Ser Glu Asn Gly Trp Thr
225                 230                 235                 240
Val Asp Ile Thr Arg Asn Phe Ser Asn Ile Ile Gln Gly Ser Gln Ile
                245                 250                 255
Ser Ser Leu Ile Leu Lys His His Ile Met Gly Pro Gly Phe Gly Phe
            260                 265                 270
Gln Asn Ile Arg Asp Pro Asp Gln Ser Thr Phe Ala Ser Leu Ala Arg
        275                 280                 285
Ser Ser Val Leu Gln Leu Asp Leu Ser His Gly Phe Ile Phe Ser Leu
    290                 295                 300
Asn Pro Arg Leu Phe Gly Thr Leu Lys Asp Leu Lys Met Leu Asn Leu
305                 310                 315                 320
Ala Phe Asn Lys Ile Asn Lys Ile Gly Glu Asn Ala Phe Tyr Gly Leu
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Asp Ser Leu Gln Val Leu Asn Leu Ser Tyr Asn Leu Leu Gly Glu Leu
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Tyr Asn Ser Asn Phe Tyr Gly Leu Pro Arg Val Ala Tyr Val Asp Leu
        355                 360                 365
Gln Arg Asn His Ile Gly Ile Ile Gln Asp Gln Thr Phe Arg Leu Leu
    370                 375                 380
Lys Thr Leu Gln Thr Leu Asp Leu Arg Asp Asn Ala Leu Lys Ala Ile
385                 390                 395                 400
Gly Phe Ile Pro Ser Ile Gln Met Val Leu Leu Gly Gly Asn Lys Leu
                405                 410                 415
Val His Leu Pro His Ile His Phe Thr Ala Asn Phe Leu Glu Leu Ser
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Glu Asn Arg Leu Glu Asn Leu Ser Asp Leu Tyr Phe Leu Leu Arg Val
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Pro Gln Leu Gln Phe Leu Ile Leu Asn Gln Asn Arg Leu Ser Ser Cys
    450                 455                 460
Lys Ala Ala His Thr Pro Ser Glu Asn Pro Ser Leu Glu Gln Leu Phe
465                 470                 475                 480
Leu Thr Glu Asn Met Leu Gln Leu Ala Trp Glu Thr Gly Leu Cys Trp
                485                 490                 495
Asp Val Phe Gln Gly Leu Ser Arg Leu Gln Ile Leu Tyr Leu Ser Asn
            500                 505                 510
Asn Tyr Leu Asn Phe Leu Pro Pro Gly Ile Phe Asn Asp Leu Val Ala
        515                 520                 525
Leu Arg Met Leu Ser Leu Ser Ala Asn Lys Leu Thr Val Leu Ser Pro
    530                 535                 540
Gly Ser Leu Pro Ala Asn Leu Glu Ile Leu Asp Ile Ser Arg Asn Gln
545                 550                 555                 560
Leu Leu Cys Pro Asp Pro Ala Leu Phe Ser Ser Leu Arg Val Leu Asp
                565                 570                 575
Ile Thr His Asn Glu Phe Val Cys Asn Cys Glu Leu Ser Thr Phe Ile
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Ser Trp Leu Asn Gln Thr Asn Val Thr Leu Phe Gly Ser Pro Ala Asp
        595                 600                 605
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Val Tyr Cys Met Tyr Pro Asn Ser Leu Leu Gly Gly Ser Leu Tyr Asn
    610                 615                 620
Ile Ser Thr Glu Asp Cys Asp Glu Glu Glu Ala Met Arg Ser Leu Lys
625                 630                 635                 640
Phe Ser Leu Phe Ile Leu Cys Thr Val Thr Leu Thr Leu Phe Leu Val
                645                 650                 655
Ile Thr Leu Val Val Ile Lys Phe Arg Gly Ile Cys Phe Leu Cys Tyr
            660                 665                 670
Lys Thr Ile Gln Lys Leu Val Phe Lys Asp Lys Val Trp Ser Leu Glu
        675                 680                 685
Pro Gly Ala Tyr Arg Tyr Asp Ala Tyr Phe Cys Phe Ser Ser Lys Asp
    690                 695                 700
Phe Glu Trp Ala Gln Asn Ala Leu Leu Lys His Leu Asp Ala His Tyr
705                 710                 715                 720
Ser Ser Arg Asn Arg Leu Arg Leu Cys Phe Glu Glu Arg Asp Phe Ile
                725                 730                 735
Pro Gly Glu Asn His Ile Ser Asn Ile Gln Ala Ala Val Trp Gly Ser
            740                 745                 750
Arg Lys Thr Val Cys Leu Val Ser Arg His Phe Leu Lys Asp Gly Trp
        755                 760                 765
Cys Leu Glu Ala Phe Arg Tyr Ala Gln Ser Arg Ser Leu Ser Asp Leu
    770                 775                 780
Lys Ser Ile Leu Ile Val Val Val Val Gly Ser Leu Ser Gln Tyr Gln
785                 790                 795                 800
Leu Met Arg His Glu Thr Ile Arg Gly Phe Leu Gln Lys Gln Gln Tyr
                805                 810                 815
Leu Arg Trp Pro Glu Asp Leu Gln Asp Val Gly Trp Phe Leu Asp Lys
            820                 825                 830
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taagaacata ctagaagtcc cttctgctag gacaacgagg atc atg gga gac cac   715
                                                 Met Gly Asp His
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ctg gac ctt ctc cta gga gtg gtg ctc atg gcc ggt cct gtg ttt gga   763
Leu Asp Leu Leu Leu Gly Val Val Leu Met Ala Gly Pro Val Phe Gly
 5                  10                  15                  20

att cct tcc tgc tcc ttt gat ggc cga ata gcc ttt tat cgt ttc tgc   811
Ile Pro Ser Cys Ser Phe Asp Gly Arg Ile Ala Phe Tyr Arg Phe Cys
                25                  30                  35

aac ctc acc cag gtc ccc cag gtc ctc aac acc act gag agg ctc ctg   859
Asn Leu Thr Gln Val Pro Gln Val Leu Asn Thr Thr Glu Arg Leu Leu
            40                  45                  50

ctg agc ttc aac tat atc agg aca gtc act gct tca tcc ttc ccc ttt   907
Leu Ser Phe Asn Tyr Ile Arg Thr Val Thr Ala Ser Ser Phe Pro Phe
        55                  60                  65

ctg gaa cag ctg cag ctg ctg gag ctc ggg agc cag tat acc ccc ttg   955
Leu Glu Gln Leu Gln Leu Leu Glu Leu Gly Ser Gln Tyr Thr Pro Leu
    70                  75                  80

act att gac aag gag gcc ttc aga aac ctg ccc aac ctt aga atc ttg   1003
Thr Ile Asp Lys Glu Ala Phe Arg Asn Leu Pro Asn Leu Arg Ile Leu
 85                  90                  95                  100

gac ctg gga agt agt aag ata tac ttc ttg cat cca gat gct ttt cag   1051
Asp Leu Gly Ser Ser Lys Ile Tyr Phe Leu His Pro Asp Ala Phe Gln
                105                 110                 115

gga ctg ttc cat ctg ttt gaa ctt aga ctg tat ttc tgt ggt ctc tct   1099
Gly Leu Phe His Leu Phe Glu Leu Arg Leu Tyr Phe Cys Gly Leu Ser
            120                 125                 130

gat gct gta ttg aaa gat ggt tat ttc aga aat tta aag gct tta act   1147
Asp Ala Val Leu Lys Asp Gly Tyr Phe Arg Asn Leu Lys Ala Leu Thr
        135                 140                 145

cgc ttg gat cta tcc aaa aat cag att cgt agc ctt tac ctt cat cct   1195
Arg Leu Asp Leu Ser Lys Asn Gln Ile Arg Ser Leu Tyr Leu His Pro
    150                 155                 160

tca ttt ggg aag ttg aat tcc tta aag tcc ata gat ttt tcc tcc aac   1243
Ser Phe Gly Lys Leu Asn Ser Leu Lys Ser Ile Asp Phe Ser Ser Asn
165                 170                 175                 180

caa ata ttc ctt gta tgt gaa cat gag ctc gag ccc cta caa ggg aaa   1291
Gln Ile Phe Leu Val Cys Glu His Glu Leu Glu Pro Leu Gln Gly Lys
                185                 190                 195

acg ctc tcc ttt ttt agc ctc gca gct aat agc ttg tat agc aga gtc   1339
Thr Leu Ser Phe Phe Ser Leu Ala Ala Asn Ser Leu Tyr Ser Arg Val
            200                 205                 210
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tca gtg gac tgg gga aaa tgt atg aac cca ttc aga aac atg gtg ctg   1387
Ser Val Asp Trp Gly Lys Cys Met Asn Pro Phe Arg Asn Met Val Leu
        215                 220                 225

gag ata gta gat gtt tct gga aat ggc tgg aca gtg gac atc aca gga   1435
Glu Ile Val Asp Val Ser Gly Asn Gly Trp Thr Val Asp Ile Thr Gly
    230                 235                 240

aac ttt agc aat gcc atc agc aaa agc cag gcc ttc tct ttg att ctt   1483
Asn Phe Ser Asn Ala Ile Ser Lys Ser Gln Ala Phe Ser Leu Ile Leu
245                 250                 255                 260

gcc cac cac atc atg ggt gcc ggg ttt ggc ttc cat aac atc aaa gat   1531
Ala His His Ile Met Gly Ala Gly Phe Gly Phe His Asn Ile Lys Asp
                265                 270                 275

cct gac cag aac aca ttt gct ggc ctg gcc aga agt tca gtg aga cac   1579
Pro Asp Gln Asn Thr Phe Ala Gly Leu Ala Arg Ser Ser Val Arg His
            280                 285                 290

ctg gac ctt tca cat ggg ttt gtc ttc tcc ctg aac tca cga gtc ttt   1627
Leu Asp Leu Ser His Gly Phe Val Phe Ser Leu Asn Ser Arg Val Phe
        295                 300                 305

gag aca ctc aag gat ttg aag gtt ctg aac ctt gcc tac aac aag ata   1675
Glu Thr Leu Lys Asp Leu Lys Val Leu Asn Leu Ala Tyr Asn Lys Ile
    310                 315                 320

aat aag att gca gat gaa gca ttt tac gga ctt gac aac ctc caa gtt   1723
Asn Lys Ile Ala Asp Glu Ala Phe Tyr Gly Leu Asp Asn Leu Gln Val
325                 330                 335                 340

ctc aat ttg tca tat aac ctt ctg ggg gaa ctt tgc agt tcg aat ttc   1771
Leu Asn Leu Ser Tyr Asn Leu Leu Gly Glu Leu Cys Ser Ser Asn Phe
                345                 350                 355

tat gga cta cct aag gta gcc tac att gat ttg caa aag aat cac att   1819
Tyr Gly Leu Pro Lys Val Ala Tyr Ile Asp Leu Gln Lys Asn His Ile
            360                 365                 370

gca ata att caa gac caa aca ttc aaa ttc ctg gaa aaa tta cag acc   1867
Ala Ile Ile Gln Asp Gln Thr Phe Lys Phe Leu Glu Lys Leu Gln Thr
        375                 380                 385

ttg gat ctc cga gac aat gct ctt aca acc att cat ttt att cca agc   1915
Leu Asp Leu Arg Asp Asn Ala Leu Thr Thr Ile His Phe Ile Pro Ser
    390                 395                 400

ata ccc gat atc ttc ttg agt ggc aat aaa cta gtg act ttg cca aag   1963
Ile Pro Asp Ile Phe Leu Ser Gly Asn Lys Leu Val Thr Leu Pro Lys
405                 410                 415                 420

atc aac ctt aca gcg aac ctc atc cac tta tca gaa aac agg cta gaa   2011
Ile Asn Leu Thr Ala Asn Leu Ile His Leu Ser Glu Asn Arg Leu Glu
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                425                 430                 435

aat cta gat att ctc tac ttt ctc cta cgg gta cct cat ctc cag att   2059
Asn Leu Asp Ile Leu Tyr Phe Leu Leu Arg Val Pro His Leu Gln Ile
            440                 445                 450

ctc att tta aat caa aat cgc ttc tcc tcc tgt agt gga gat caa acc   2107
Leu Ile Leu Asn Gln Asn Arg Phe Ser Ser Cys Ser Gly Asp Gln Thr
        455                 460                 465

cct tca gag aat ccc agc tta gaa cag ctt ttc ctt gga gaa aat atg   2155
Pro Ser Glu Asn Pro Ser Leu Glu Gln Leu Phe Leu Gly Glu Asn Met
    470                 475                 480

ttg caa ctt gcc tgg gaa act gag ctc tgt tgg gat gtt ttt gag gga   2203
Leu Gln Leu Ala Trp Glu Thr Glu Leu Cys Trp Asp Val Phe Glu Gly
485                 490                 495                 500

ctt tct cat ctt caa gtt ctg tat ttg aat cat aac tat ctt aat tcc   2251
Leu Ser His Leu Gln Val Leu Tyr Leu Asn His Asn Tyr Leu Asn Ser
                505                 510                 515

ctt cca cca gga gta ttt agc cat ctg act gca tta agg gga cta agc   2299
Leu Pro Pro Gly Val Phe Ser His Leu Thr Ala Leu Arg Gly Leu Ser
            520                 525                 530

ctc aac tcc aac agg ctg aca gtt ctt tct cac aat gat tta cct gct   2347
Leu Asn Ser Asn Arg Leu Thr Val Leu Ser His Asn Asp Leu Pro Ala
        535                 540                 545

aat tta gag atc ctg gac ata tcc agg aac cag ctc cta gct cct aat   2395
Asn Leu Glu Ile Leu Asp Ile Ser Arg Asn Gln Leu Leu Ala Pro Asn
    550                 555                 560

cct gat gta ttt gta tca ctt agt gtc ttg gat ata act cat aac aag   2443
Pro Asp Val Phe Val Ser Leu Ser Val Leu Asp Ile Thr His Asn Lys
565                 570                 575                 580

ttc att tgt gaa tgt gaa ctt agc act ttt atc aat tgg ctt aat cac   2491
Phe Ile Cys Glu Cys Glu Leu Ser Thr Phe Ile Asn Trp Leu Asn His
                585                 590                 595

acc aat gtc act ata gct ggg cct cct gca gac ata tat tgt gtg tac   2539
Thr Asn Val Thr Ile Ala Gly Pro Pro Ala Asp Ile Tyr Cys Val Tyr
            600                 605                 610

cct gac tcg ctc tct ggg gtt tcc ctc ttc tct ctt tcc acg gaa ggt   2587
Pro Asp Ser Leu Ser Gly Val Ser Leu Phe Ser Leu Ser Thr Glu Gly
        615                 620                 625

tgt gat gaa gag gaa gtc tta aag tcc cta aag ttc tcc ctt ttc att   2635
Cys Asp Glu Glu Glu Val Leu Lys Ser Leu Lys Phe Ser Leu Phe Ile
    630                 635                 640

gta tgc act gtc act ctg act ctg ttc ctc atg acc atc ctc aca gtc   2683
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Val Cys Thr Val Thr Leu Thr Leu Phe Leu Met Thr Ile Leu Thr Val
645                 650                 655                 660

aca aag ttc cgg ggc ttc tgt ttt atc tgt tat aag aca gcc cag aga   2731
Thr Lys Phe Arg Gly Phe Cys Phe Ile Cys Tyr Lys Thr Ala Gln Arg
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ctg gtg ttc aag gac cat ccc cag ggc aca gaa cct gat atg tac aaa   2779
Leu Val Phe Lys Asp His Pro Gln Gly Thr Glu Pro Asp Met Tyr Lys
            680                 685                 690

tat gat gcc tat ttg tgc ttc agc agc aaa gac ttc aca tgg gtg cag   2827
Tyr Asp Ala Tyr Leu Cys Phe Ser Ser Lys Asp Phe Thr Trp Val Gln
        695                 700                 705

aat gct ttg ctc aaa cac ctg gac act caa tac agt gac caa aac aga   2875
Asn Ala Leu Leu Lys His Leu Asp Thr Gln Tyr Ser Asp Gln Asn Arg
    710                 715                 720

ttc aac ctg tgc ttt gaa gaa aga gac ttt gtc cca gga gaa aac cgc   2923
Phe Asn Leu Cys Phe Glu Glu Arg Asp Phe Val Pro Gly Glu Asn Arg
725                 730                 735                 740

att gcc aat atc cag gat gcc atc tgg aac agt aga aag atc gtt tgt   2971
Ile Ala Asn Ile Gln Asp Ala Ile Trp Asn Ser Arg Lys Ile Val Cys
                745                 750                 755

ctt gtg agc aga cac ttc ctt aga gat ggc tgg tgc ctt gaa gcc ttc   3019
Leu Val Ser Arg His Phe Leu Arg Asp Gly Trp Cys Leu Glu Ala Phe
            760                 765                 770

agt tat gcc cag ggc agg tgc tta tct gac ctt aac agt gct ctc atc   3067
Ser Tyr Ala Gln Gly Arg Cys Leu Ser Asp Leu Asn Ser Ala Leu Ile
        775                 780                 785

atg gtg gtg gtt ggg tcc ttg tcc cag tac cag ttg atg aaa cat caa   3115
Met Val Val Val Gly Ser Leu Ser Gln Tyr Gln Leu Met Lys His Gln
    790                 795                 800

tcc atc aga ggc ttt gta cag aaa cag cag tat ttg agg tgg cct gag   3163
Ser Ile Arg Gly Phe Val Gln Lys Gln Gln Tyr Leu Arg Trp Pro Glu
805                 810                 815                 820

gat ctc cag gat gtt ggc tgg ttt ctt cat aaa ctc tct caa cag ata   3211
Asp Leu Gln Asp Val Gly Trp Phe Leu His Lys Leu Ser Gln Gln Ile
                825                 830                 835

cta aag aaa gaa aaa gaa aag aag aaa gac aat aac att ccg ttg caa   3259
Leu Lys Lys Glu Lys Glu Lys Lys Lys Asp Asn Asn Ile Pro Leu Gln
            840                 845                 850

act gta gca acc atc tcc taatcaaagg agcaatttcc aacttatctc          3307
Thr Val Ala Thr Ile Ser
        855
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aagccacaaa taactcttca ctttgtattt gcaccaagtt atcattttgg ggtcctctct 3367
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Tyr Arg Phe Cys Asn Leu Thr Gln Val Pro Gln Val Leu Asn Thr Thr
        35                  40                  45
Glu Arg Leu Leu Leu Ser Phe Asn Tyr Ile Arg Thr Val Thr Ala Ser
    50                  55                  60
Ser Phe Pro Phe Leu Glu Gln Leu Gln Leu Leu Glu Leu Gly Ser Gln
65                  70                  75                  80
Tyr Thr Pro Leu Thr Ile Asp Lys Glu Ala Phe Arg Asn Leu Pro Asn
                85                  90                  95
Leu Arg Ile Leu Asp Leu Gly Ser Ser Lys Ile Tyr Phe Leu His Pro
            100                 105                 110
Asp Ala Phe Gln Gly Leu Phe His Leu Phe Glu Leu Arg Leu Tyr Phe
        115                 120                 125
Cys Gly Leu Ser Asp Ala Val Leu Lys Asp Gly Tyr Phe Arg Asn Leu
    130                 135                 140
Lys Ala Leu Thr Arg Leu Asp Leu Ser Lys Asn Gln Ile Arg Ser Leu
145                 150                 155                 160
Tyr Leu His Pro Ser Phe Gly Lys Leu Asn Ser Leu Lys Ser Ile Asp
                165                 170                 175
Phe Ser Ser Asn Gln Ile Phe Leu Val Cys Glu His Glu Leu Glu Pro
            180                 185                 190
Leu Gln Gly Lys Thr Leu Ser Phe Phe Ser Leu Ala Ala Asn Ser Leu
        195                 200                 205
Tyr Ser Arg Val Ser Val Asp Trp Gly Lys Cys Met Asn Pro Phe Arg
    210                 215                 220
Asn Met Val Leu Glu Ile Val Asp Val Ser Gly Asn Gly Trp Thr Val
225                 230                 235                 240
Asp Ile Thr Gly Asn Phe Ser Asn Ala Ile Ser Lys Ser Gln Ala Phe
                245                 250                 255
Ser Leu Ile Leu Ala His His Ile Met Gly Ala Gly Phe Gly Phe His
            260                 265                 270
Asn Ile Lys Asp Pro Asp Gln Asn Thr Phe Ala Gly Leu Ala Arg Ser
        275                 280                 285
Ser Val Arg His Leu Asp Leu Ser His Gly Phe Val Phe Ser Leu Asn
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Ser Arg Val Phe Glu Thr Leu Lys Asp Leu Lys Val Leu Asn Leu Ala
305                 310                 315                 320
Tyr Asn Lys Ile Asn Lys Ile Ala Asp Glu Ala Phe Tyr Gly Leu Asp
                325                 330                 335
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385                 390                 395                 400
Phe Ile Pro Ser Ile Pro Asp Ile Phe Leu Ser Gly Asn Lys Leu Val
                405                 410                 415
Thr Leu Pro Lys Ile Asn Leu Thr Ala Asn Leu Ile His Leu Ser Glu
            420                 425                 430
Asn Arg Leu Glu Asn Leu Asp Ile Leu Tyr Phe Leu Leu Arg Val Pro
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His Leu Gln Ile Leu Ile Leu Asn Gln Asn Arg Phe Ser Ser Cys Ser
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465                 470                 475                 480
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Val Phe Glu Gly Leu Ser His Leu Gln Val Leu Tyr Leu Asn His Asn
            500                 505                 510
Tyr Leu Asn Ser Leu Pro Pro Gly Val Phe Ser His Leu Thr Ala Leu
        515                 520                 525
Arg Gly Leu Ser Leu Asn Ser Asn Arg Leu Thr Val Leu Ser His Asn
    530                 535                 540
Asp Leu Pro Ala Asn Leu Glu Ile Leu Asp Ile Ser Arg Asn Gln Leu
545                 550                 555                 560
Leu Ala Pro Asn Pro Asp Val Phe Val Ser Leu Ser Val Leu Asp Ile
                565                 570                 575
Thr His Asn Lys Phe Ile Cys Glu Cys Glu Leu Ser Thr Phe Ile Asn
            580                 585                 590
Trp Leu Asn His Thr Asn Val Thr Ile Ala Gly Pro Pro Ala Asp Ile
        595                 600                 605
Tyr Cys Val Tyr Pro Asp Ser Leu Ser Gly Val Ser Leu Phe Ser Leu
    610                 615                 620
Ser Thr Glu Gly Cys Asp Glu Glu Glu Val Leu Lys Ser Leu Lys Phe
625                 630                 635                 640
Ser Leu Phe Ile Val Cys Thr Val Thr Leu Thr Leu Phe Leu Met Thr
                645                 650                 655
Ile Leu Thr Val Thr Lys Phe Arg Gly Phe Cys Phe Ile Cys Tyr Lys
            660                 665                 670
Thr Ala Gln Arg Leu Val Phe Lys Asp His Pro Gln Gly Thr Glu Pro
        675                 680                 685
Asp Met Tyr Lys Tyr Asp Ala Tyr Leu Cys Phe Ser Ser Lys Asp Phe
    690                 695                 700
Thr Trp Val Gln Asn Ala Leu Leu Lys His Leu Asp Thr Gln Tyr Ser
705                 710                 715                 720
Asp Gln Asn Arg Phe Asn Leu Cys Phe Glu Glu Arg Asp Phe Val Pro
                725                 730                 735
Gly Glu Asn Arg Ile Ala Asn Ile Gln Asp Ala Ile Trp Asn Ser Arg
            740                 745                 750
Lys Ile Val Cys Leu Val Ser Arg His Phe Leu Arg Asp Gly Trp Cys
        755                 760                 765
Leu Glu Ala Phe Ser Tyr Ala Gln Gly Arg Cys Leu Ser Asp Leu Asn
    770                 775                 780
Ser Ala Leu Ile Met Val Val Val Gly Ser Leu Ser Gln Tyr Gln Leu
785                 790                 795                 800
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Met Lys His Gln Ser Ile Arg Gly Phe Val Gln Lys Gln Gln Tyr Leu
                805                 810                 815
Arg Trp Pro Glu Asp Leu Gln Asp Val Gly Trp Phe Leu His Lys Leu
            820                 825                 830
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 1               5                  10                  15

ctg tca caa ctc cta gct gtc act caa ggg aag acg ctg gtg ctg ggg   96
Leu Ser Gln Leu Leu Ala Val Thr Gln Gly Lys Thr Leu Val Leu Gly
            20                  25                  30

aag gaa ggg gaa tca gca gaa ctg ccc tgc gag agt tcc cag aag aag   144
Lys Glu Gly Glu Ser Ala Glu Leu Pro Cys Glu Ser Ser Gln Lys Lys
        35                  40                  45

atc aca gtc ttc acc tgg aag ttc tct gac cag agg aag att ctg ggg   192
Ile Thr Val Phe Thr Trp Lys Phe Ser Asp Gln Arg Lys Ile Leu Gly
    50                  55                  60

cag cat ggc aaa ggt gta tta att aga gga ggt tcg cct tcg cag ttt   240
Gln His Gly Lys Gly Val Leu Ile Arg Gly Gly Ser Pro Ser Gln Phe
65                  70                  75                  80

gat cgt ttt gat tcc aaa aaa ggg gca tgg gag aaa gga tcg ttt cct   288
Asp Arg Phe Asp Ser Lys Lys Gly Ala Trp Glu Lys Gly Ser Phe Pro
                85                  90                  95

ctc atc atc aat aaa ctt aag atg gaa gac tct cag act tat atc tgt   336
Leu Ile Ile Asn Lys Leu Lys Met Glu Asp Ser Gln Thr Tyr Ile Cys
            100                 105                 110

gag ctg gag aac agg aaa gag gag gtg gag ttg tgg gtg ttc aaa gtg   384
Glu Leu Glu Asn Arg Lys Glu Glu Val Glu Leu Trp Val Phe Lys Val
        115                 120                 125

acc ttc agt ccg ggt acc agc ctg ttg caa ggg cag agc ctg acc ctg   432
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Thr Phe Ser Pro Gly Thr Ser Leu Leu Gln Gly Gln Ser Leu Thr Leu
    130                 135                 140

acc ttg gat agc aac tct aag gtc tct aac ccc ttg aca gag tgc aaa   480
Thr Leu Asp Ser Asn Ser Lys Val Ser Asn Pro Leu Thr Glu Cys Lys
145                 150                 155                 160

cac aaa aag ggt aaa gtt gtc agt ggt tcc aaa gtt ctc tcc atg tcc   528
His Lys Lys Gly Lys Val Val Ser Gly Ser Lys Val Leu Ser Met Ser
                165                 170                 175

aac cta agg gtt cag gac agc gac ttc tgg aac tgc acc gtg acc ctg   576
Asn Leu Arg Val Gln Asp Ser Asp Phe Trp Asn Cys Thr Val Thr Leu
            180                 185                 190

gac cag aaa aag aac tgg ttc ggc atg aca ctc tca gtg ctg ggt ttt   624
Asp Gln Lys Lys Asn Trp Phe Gly Met Thr Leu Ser Val Leu Gly Phe
        195                 200                 205

cag agc aca gct atc acg gcc tat aag agt gag gga gag tca gcg gag   672
Gln Ser Thr Ala Ile Thr Ala Tyr Lys Ser Glu Gly Glu Ser Ala Glu
    210                 215                 220

ttc tcc ttc cca ctc aac ttt gca gag gaa aac ggg tgg gga gag ctg   720
Phe Ser Phe Pro Leu Asn Phe Ala Glu Glu Asn Gly Trp Gly Glu Leu
225                 230                 235                 240

atg tgg aag gca gag aag gat tct ttc ttc cag ccc tgg atc tcc ttc   768
Met Trp Lys Ala Glu Lys Asp Ser Phe Phe Gln Pro Trp Ile Ser Phe
                245                 250                 255

tcc ata aag aac aaa gag gtg tcc gta caa aag tcc acc aaa gac ctc   816
Ser Ile Lys Asn Lys Glu Val Ser Val Gln Lys Ser Thr Lys Asp Leu
            260                 265                 270

aag ctc cag ctg aag gaa acg ctc cca ctc acc ctc aag ata ccc cag   864
Lys Leu Gln Leu Lys Glu Thr Leu Pro Leu Thr Leu Lys Ile Pro Gln
        275                 280                 285

gtc tcg ctt cag ttt gct ggt tct ggc aac ctg act ctg act ctg gac   912
Val Ser Leu Gln Phe Ala Gly Ser Gly Asn Leu Thr Leu Thr Leu Asp
    290                 295                 300

aaa ggg aca ctg cat cag gaa gtg aac ctg gtg gtg atg aaa gtg gct   960
Lys Gly Thr Leu His Gln Glu Val Asn Leu Val Val Met Lys Val Ala
305                 310                 315                 320

cag ctc aac aat act ttg acc tgt gag gtg atg gga cct acc tct ccc  1008
Gln Leu Asn Asn Thr Leu Thr Cys Glu Val Met Gly Pro Thr Ser Pro
                325                 330                 335

aag atg aga ctg acc ctg aag cag gag aac cag gag gcc agg gtc tct  1056
Lys Met Arg Leu Thr Leu Lys Gln Glu Asn Gln Glu Ala Arg Val Ser
            340                 345                 350
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        355                 360                 365

cag tgt cta ctg agt gaa ggt gat aag gtc aag atg gac tcc agg atc   1152
Gln Cys Leu Leu Ser Glu Gly Asp Lys Val Lys Met Asp Ser Arg Ile
    370                 375                 380

cag gtt tta tcc aga ggg gtg tac cag ttc tcc ctt ttc att gta tgc   1200
Gln Val Leu Ser Arg Gly Val Tyr Gln Phe Ser Leu Phe Ile Val Cys
385                 390                 395                 400

act gtc act ctg act ctg ttc ctc atg acc atc ctc aca gtc aca aag   1248
Thr Val Thr Leu Thr Leu Phe Leu Met Thr Ile Leu Thr Val Thr Lys
                405                 410                 415

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Phe Arg Gly Phe Cys Phe Ile Cys Tyr Lys Thr Ala Gln Arg Leu Val
            420                 425                 430

ttc aag gac cat ccc cag ggc aca gaa cct gat atg tac aaa tat gat   1344
Phe Lys Asp His Pro Gln Gly Thr Glu Pro Asp Met Tyr Lys Tyr Asp
        435                 440                 445

gcc tat ttg tgc ttc agc agc aaa gac ttc aca tgg gtg cag aat gct   1392
Ala Tyr Leu Cys Phe Ser Ser Lys Asp Phe Thr Trp Val Gln Asn Ala
    450                 455                 460

ttg ctc aaa cac ctg gac act caa tac agt gac caa aac aga ttc aac   1440
Leu Leu Lys His Leu Asp Thr Gln Tyr Ser Asp Gln Asn Arg Phe Asn
465                 470                 475                 480

ctg tgc ttt gaa gaa aga gac ttt gtc cca gga gaa aac cgc att gcc   1488
Leu Cys Phe Glu Glu Arg Asp Phe Val Pro Gly Glu Asn Arg Ile Ala
                485                 490                 495

aat atc cag gat gcc atc tgg aac agt aga aag atc gtt tgt ctt gtg   1536
Asn Ile Gln Asp Ala Ile Trp Asn Ser Arg Lys Ile Val Cys Leu Val
            500                 505                 510

agc aga cac ttc ctt aga gat ggc tgg tgc ctt gaa gcc ttc agt tat   1584
Ser Arg His Phe Leu Arg Asp Gly Trp Cys Leu Glu Ala Phe Ser Tyr
        515                 520                 525

gcc cag ggc agg tgc tta tct gac ctt aac agt gct ctc atc atg gtg   1632
Ala Gln Gly Arg Cys Leu Ser Asp Leu Asn Ser Ala Leu Ile Met Val
    530                 535                 540

gtg gtt ggg tcc ttg tcc cag tac cag ttg atg aaa cat caa tcc atc   1680
Val Val Gly Ser Leu Ser Gln Tyr Gln Leu Met Lys His Gln Ser Ile
545                 550                 555                 560

aga ggc ttt gta cag aaa cag cag tat ttg agg tgg cct gag gat ctc   1728
Arg Gly Phe Val Gln Lys Gln Gln Tyr Leu Arg Trp Pro Glu Asp Leu
                565                 570                 575
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            580                 585                 590

aaa gaa aaa gaa aag aag aaa gac aat aac att ccg ttg caa act gta   1824
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        595                 600                 605

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            20                  25                  30
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        35                  40                  45
Ile Thr Val Phe Thr Trp Lys Phe Ser Asp Gln Arg Lys Ile Leu Gly
    50                  55                  60
Gln His Gly Lys Gly Val Leu Ile Arg Gly Gly Ser Pro Ser Gln Phe
65                  70                  75                  80
Asp Arg Phe Asp Ser Lys Lys Gly Ala Trp Glu Lys Gly Ser Phe Pro
                85                  90                  95
Leu Ile Ile Asn Lys Leu Lys Met Glu Asp Ser Gln Thr Tyr Ile Cys
            100                 105                 110
Glu Leu Glu Asn Arg Lys Glu Glu Val Glu Leu Trp Val Phe Lys Val
        115                 120                 125
Thr Phe Ser Pro Gly Thr Ser Leu Leu Gln Gly Gln Ser Leu Thr Leu
    130                 135                 140
Thr Leu Asp Ser Asn Ser Lys Val Ser Asn Pro Leu Thr Glu Cys Lys
145                 150                 155                 160
His Lys Lys Gly Lys Val Val Ser Gly Ser Lys Val Leu Ser Met Ser
                165                 170                 175
Asn Leu Arg Val Gln Asp Ser Asp Phe Trp Asn Cys Thr Val Thr Leu
            180                 185                 190
Asp Gln Lys Lys Asn Trp Phe Gly Met Thr Leu Ser Val Leu Gly Phe
        195                 200                 205
Gln Ser Thr Ala Ile Thr Ala Tyr Lys Ser Glu Gly Glu Ser Ala Glu
    210                 215                 220
Phe Ser Phe Pro Leu Asn Phe Ala Glu Glu Asn Gly Trp Gly Glu Leu
225                 230                 235                 240
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                245                 250                 255
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Ser Ile Lys Asn Lys Glu Val Ser Val Gln Lys Ser Thr Lys Asp Leu
                260                 265                 270
Lys Leu Gln Leu Lys Glu Thr Leu Pro Leu Thr Leu Lys Ile Pro Gln
        275                 280                 285
Val Ser Leu Gln Phe Ala Gly Ser Gly Asn Leu Thr Leu Thr Leu Asp
    290                 295                 300
Lys Gly Thr Leu His Gln Glu Val Asn Leu Val Val Met Lys Val Ala
305                 310                 315                 320
Gln Leu Asn Asn Thr Leu Thr Cys Glu Val Met Gly Pro Thr Ser Pro
                325                 330                 335
Lys Met Arg Leu Thr Leu Lys Gln Glu Asn Gln Glu Ala Arg Val Ser
            340                 345                 350
Glu Glu Gln Lys Val Val Gln Val Val Ala Pro Glu Thr Gly Leu Trp
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Gln Cys Leu Leu Ser Glu Gly Asp Lys Val Lys Met Asp Ser Arg Ile
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Gln Val Leu Ser Arg Gly Val Tyr Gln Phe Ser Leu Phe Ile Val Cys
385                 390                 395                 400
Thr Val Thr Leu Thr Leu Phe Leu Met Thr Ile Leu Thr Val Thr Lys
                405                 410                 415
Phe Arg Gly Phe Cys Phe Ile Cys Tyr Lys Thr Ala Gln Arg Leu Val
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Phe Lys Asp His Pro Gln Gly Thr Glu Pro Asp Met Tyr Lys Tyr Asp
        435                 440                 445
Ala Tyr Leu Cys Phe Ser Ser Lys Asp Phe Thr Trp Val Gln Asn Ala
    450                 455                 460
Leu Leu Lys His Leu Asp Thr Gln Tyr Ser Asp Gln Asn Arg Phe Asn
465                 470                 475                 480
Leu Cys Phe Glu Glu Arg Asp Phe Val Pro Gly Glu Asn Arg Ile Ala
                485                 490                 495
Asn Ile Gln Asp Ala Ile Trp Asn Ser Arg Lys Ile Val Cys Leu Val
            500                 505                 510
Ser Arg His Phe Leu Arg Asp Gly Trp Cys Leu Glu Ala Phe Ser Tyr
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Ala Gln Gly Arg Cys Leu Ser Asp Leu Asn Ser Ala Leu Ile Met Val
    530                 535                 540
Val Val Gly Ser Leu Ser Gln Tyr Gln Leu Met Lys His Gln Ser Ile
545                 550                 555                 560
Arg Gly Phe Val Gln Lys Gln Gln Tyr Leu Arg Trp Pro Glu Asp Leu
                565                 570                 575
Gln Asp Val Gly Trp Phe Leu His Lys Leu Ser Gln Gln Ile Leu Lys
            580                 585                 590
Lys Glu Lys Glu Lys Lys Lys Asp Asn Asn Ile Pro Leu Gln Thr Val
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Ala Thr Ile Ser
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INTERNATIONAL SEARCH REPORT

	ational Application No
	PCT/US 01/22978

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/255 C12N15/31 A61K39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEWTON S M C ET AL: "IMMUNE RESPONSE TO CHOLERA TOXIN EPITOPE INSERTED IN SALMONELLA FLAGELLIN" SCIENCE (WASHINGTON D C), vol. 244, no. 4900, 1989, pages 70-72, XP001094029 ISSN: 0036-8075 the whole document -/--	1-6, 11-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 August 2002	11/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bilang, J

INTERNATIONAL SEARCH REPORT

	tional Application No
	PCT/US 01/22978

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MCSORLEY S J ET AL: "Characterization of CD4+ T cell responses during natural infection with Salmonella typhimurium." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD.: 1950) UNITED STATES 15 JAN 2000, vol. 164, no. 2, 15 January 2000 (2000-01-15), pages 986-993, XP002210840 ISSN: 0022-1767 the whole document	1-6,11, 12,15, 16,19-22
X	EAVES-PYLES T ET AL: "Flagellin, a novel mediator of Salmonella-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD.: 1950) UNITED STATES 15 JAN 2001, vol. 166, no. 2, 15 January 2001 (2001-01-15), pages 1248-1260, XP002210841 ISSN: 0022-1767 the whole document	1-6,11, 12,15, 16,19-22
X	GEWIRTZ ANDREW T ET AL: "Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response." JOURNAL OF CLINICAL INVESTIGATION, vol. 107, no. 1, January 2001 (2001-01), pages 99-109, XP002210842 ISSN: 0021-9738 the whole document	1-6,11, 12,15, 16,19-22
A	FELIX GEORG ET AL: "Plants have a sensitive perception system for the most conserved domain of bacterial flagellin" PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 18, no. 3, May 1999 (1999-05), pages 265-276, XP002148873 ISSN: 0960-7412	
P,X	HAYASHI FUMITAKA ET AL: "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." NATURE (LONDON), vol. 410, no. 6832, 2001, pages 1099-1103, XP002210843 ISSN: 0028-0836 the whole document	1-35

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
E	WO 02 09748 A (MEDZHITOV RUSLAN M PH D ;UNIV YALE (US)) 7 February 2002 (2002-02-07) page 51, line 21 -page 52, line 11	5,6, 11-22

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

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I itional Application No
PCT/US 01/22978

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		AU 8640501 A	13-02-2002
		WO 0209748 A1	07-02-2002